HUMAN INSULIN

EFFICACY AND IMMUNOGENICITY

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HUMAN INSULIN:
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voor mijn dochter,
Anke en Wilh.

The cover represents a detail from 'The temptation of St. Anthony', a subject Hieronymus Bosch frequently depicted. It should be seen in connection with the Anthonite order, wich had founded hospitals in the Middle Ages. These works were often placed in the rooms of the sick, so that they could contemplate the Saint, resisting temptation, and repent.

CONTENTS

Chapter 1.

REVIEW AND INTRODUCTION

Introduction.

The first patient to be treated with insulin was Leonard Thompson, a 14 year old boy from Toronto, Canada. On January 11, 1922 he received a crude extract of an animal pancreas prepared by Banting and Best (1). Since then the extraction process from animal pancreas has been greatly improved and, as the result of these efforts, preparations with very low impurity levels are available since the mid seventies. However the animal species has not been changed since 1922 untill recently. First there has been a development to use exclusively porcine insulin (PI) and no more beef insulin or beef/pork mixtures. This shift has been caused by the fact that PI differs from human insulin (HI) in only one aminoacid, whereas beef insulin differs in 3 aminoacids, and thus is more immunogenic than PI in man. The second important development has been the recent introduction of human insulin. Already in 1960, the aminoacids sequence of human insulin had been mapped (2). Using these data it was possible to make fully synthetic HI in small quantities. However, the chemical synthesis of insulin on a commercial scale was not feasible, so that other ways to produce HI had to be investigated. Now two different processes are available: the transformation of PI to HI (semisynthetic HI) and the production of insulin by E. Coli bacteria using the recombinant DNA method (biosynthetic HI). These production processes will be explained briefly on the following pages. It was of course necessary to examine in detail the properties of human insulin and to compare the effects of HI with those of PI. This thesis is an attribution to these studies. After a summary of the studies by other investigators, it describes first the method we used to analyse insulin binding antibodies and then three clinical studies comparing efficacy and immunogenicity of human and porcine insulin.

Production of semisynthetic human insulin.

Alanine is the aminoacid in position 30 of the B chain of PI. In HI this position is occupied by threonine. The remaining aminoacids sequence of

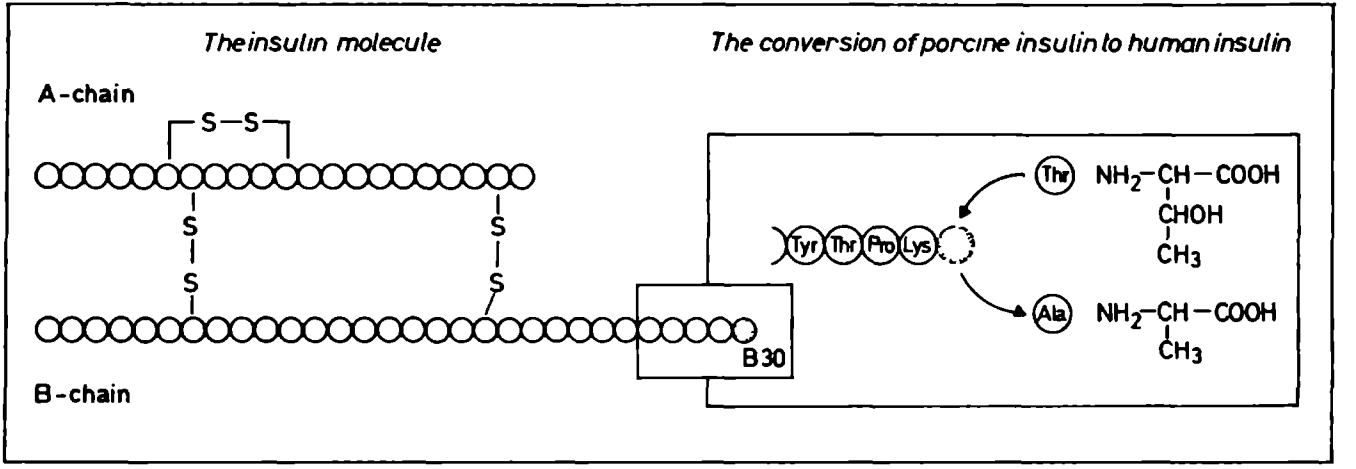

Fig. 1. Production process of semsynthetic human insulin.
The only difference between HI and PI is in the B30 position of the molecule. By replacement of Alanine by Threonine in this position, porcine insulin is converted to human insulin.

the A and B chains of these insulins is identical. In 1966 the first attempt was made to transform PI to HI (3). Although the production process was not completely succesfull, it was patented anyway. It lasted until 1978 before the optimum conditions for this conversion were found (4,5). To water, containing a high concentration of a distinct inert organic substance and trypsine as the enzyme, porcine insulin and a surplus of threonine ester is added. Under these conditions alanine in the B30 position of the PI molecule is replaced by threonine (fig. 1). Using this method on PI which is not yet purified chromatographically, and thus contains some proinsulin, the loss of about 3% of PI is almost compensated because trypsin cleaves proinsulin into insulin and C-peptide. After this procedure the preparation is purified chromotographically. The impurities consisting of trypsin, proinsulin, glucagon and other peptides are below 1 part per million in the final preparation. The contamination of PI in the semisynthetic HI preparation is less than 0.1%. In 1982 HI was produced for the first time in this way on a commercial scale (6,7).

Production of biosynthetic HI.

In 1978 Crea et al. (8) published a study on the chemical synthesis of HI genes. They prepared separate genes for the aminoacid sequences of the A and B chains of human insulin. The DNA sequences were biochemically interposed in plasmid rings, circles of DNA in bacteria which are reduplicated. By this method considerable amounts of DNA sequences for A and B chains were produced. After coupling these sequences to genes of galactosidase, Goeddel et al. (9) implanted these new genes into plasmid rings of E Coli. By bringing these bacteria in a medium with a high concentration of galactose and a low concentration of glucose, they are forced to make large quantities of galactosidase and thus also A and B chains of insulin. After the bacteria have been loaded with protein the membranes are destroyed and the A and B chains in the medium are isolated and detached from the galactosidase molecule. The synthesis of A and B chains into insulin by Goeddel et al. elded 10-15% of HI (9) because many mismatches of A and B chains are possible. In recent production processes this yield has been improved up to 50 to 60% (10) and a further improve-

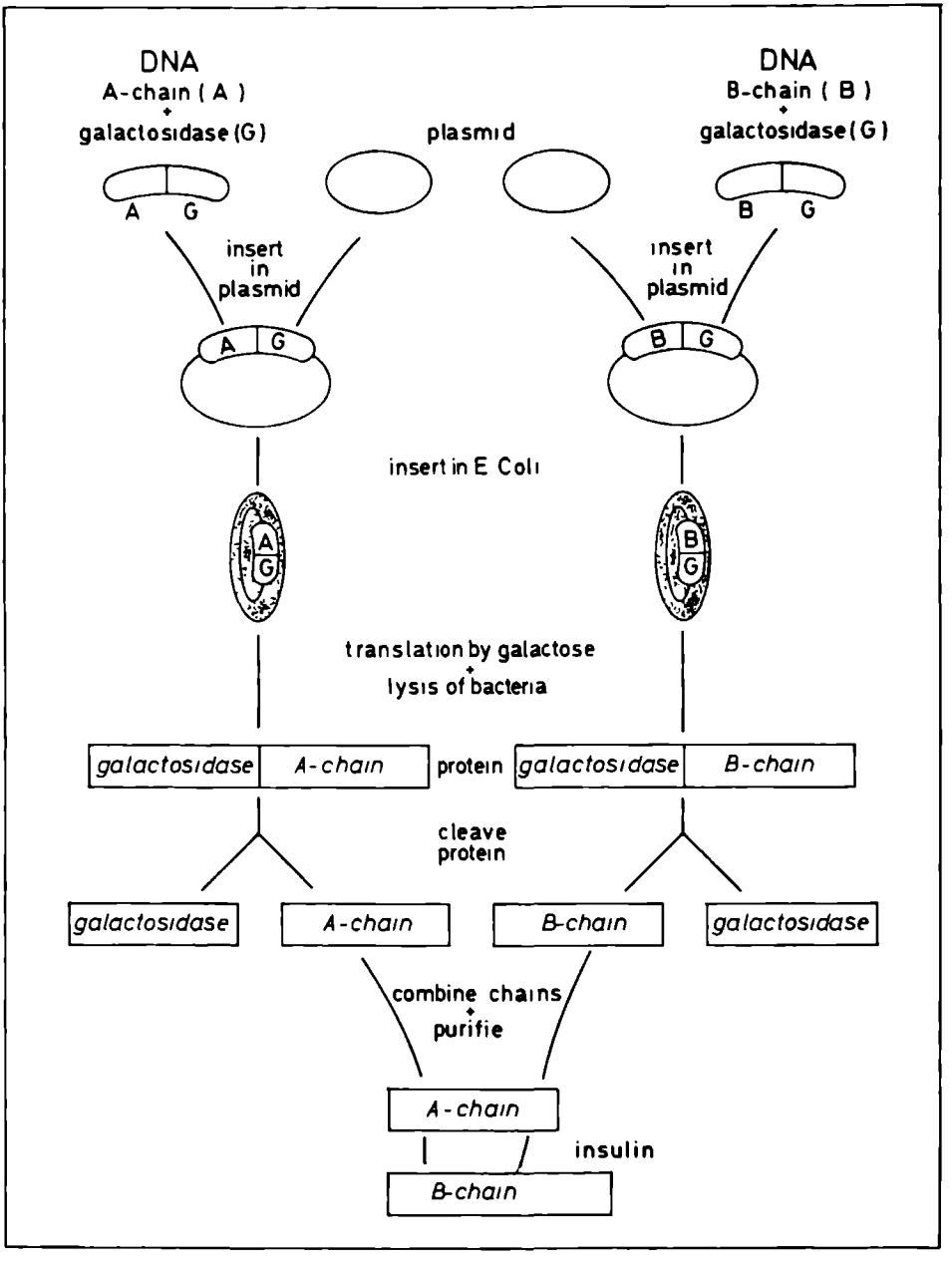

Fig. 2. Production process of biosynthetic human insulin.
The consecutive production steps from A and B chain DNA to human insulin are displayed. For a complete explanation see text.

ment can be expected in the near future. Figure 2 shows the different steps of the production process. After the synthesis insulin is purified chromatographically. Still remaining impurities must be derived from E Coli bacteria. Using a bioassay no endotoxins could be demonstrated in Ginea pigs injected with biosynthetic HI (10). With a solid phase radioimmunoassay no endotoxins could be shown in the insulin preparation above a level of 4 parts per million (10). In 10 insulin dependent diabetic patients treated with biosynthetic HI for 6 months, no significant changes in antibodies against endotoxins could be demonstrated (11). So it seems that no significant contamination by E Coli endotoxins is present in biosynthetic HI preparations, but this must be confirmed in larger clinical studies.
New developments are making the production of HI with the recombinant DNA method more economic. The first one is that proinsulin can be produced now in one step (12) and then can be converted enzymatically to insulin. Using this method it is not possible to get mismatches between separate A and B chains. The second development is that several companies are preparing a production process in which the polypeptide is not stored in the bacteria or other microorganisms, but secreted as an exotoxin, so that there is no need to destroy the microorganisms to harvest the insulin.

Behaviour and effects of HI.

The efficacy of HI has been reported in several studies. In the next paragraphs the different aspects of pharmacokinetics of HI will be discussed, with special emphasis on the difference between PI and HI for the following items: glucose levels, absorption kinetics of subcutaneously injected insulin, contraregulation of hypoglycaemias and efficacy in the treatment of diabetic ketoacidosis.

Bloodglucose lowering effects.

In 1980 Schluter et al. (13) did not find any difference in the hypoglycaemic effect between intravenously administered PI and fully synthetic HI in 12 healthy volunteers. In other studies also no difference

was found between the hypoglycaemic effects of short acting PI and semisynthetic HI (14,15,16) nor between PI and biosynthetic HI (17,18) after subcutaneous injection in small groups of healthy volunteers. In some studies, however, a significantly faster decline of blood glucose was demonstrated after subcutaneous injection of short acting semisynthetic (19) as well as of short acting biosynthetic (20,21) HI. After intravenous administration, however, both insulins were equally effective (19,21).

In several clinical studies comparing HI and PI in diabetic patients slight differences were found in home-monitored blood glucose using the same farmacological formulation and dose. Castillo et al. obtained lower blood glucose levels at 8.30 p.m. in patients treated with two injections of human Monotard and Actrapid insulin (22). Greene et al. found lower blood glucose levels after lunch during HI (23). In a multicentre trial Home et al. (24) observed higher prebreakfast glucose levels during HI. In other studies, however, no significant differences were found (25,26).

A possible explanation for these differences could be the behaviour of insulin after subcutaneous injection. In some studies in healthy volunteers the absorption of human insulin, studied by plasma free insulin levels after subcutaneous injection of insulins of the same pharmacological formulation, was slightly faster than the absorption of PI (27,28) but several other studies showed no difference (25,29,30,31,32, 33).

Since no important differences in blood glucose values were found in the clinical studies mentioned, the different absorption of HI and PI from subcutaneous tissue, if really existing, is probably not of significant clinical importance.

Counterregulatory responses to hypoglycaemias.

After i.v. administration of the same amounts of HI and PI in healthy volunteers similar decreases of blood glucose were observed in several studies (13,31,34,35). Schluter et al. found identical patterns of counterregulatory hormones after administration of fully synthetic HI and PI

(13). However, using the same methods, they observed no increase of growth hormone and a smaller reaction of cortisol after a hypoglycaemia induced by biosynthetic HI (31). This was not confirmed by Rosak et al. (34). Also after semisynthetic HI these differences were not found (35). In clinical studies no differences in frequency of subjective hypoglycaemias were found between HI and PI (23,26,36,37,38). So the impression from these studies is that if this difference in counterregulatory effect is real, it has no consequences for the clinical situation.

Efficacy in the treatment of diabetic ketoacidosis.

If a difference in efficacy between HI and PI exists, it might be apparent more clearly during the treatment of the most severe acute complication of diabetes, ketoacidosis. So far, only one study compared the efficacy of the two insulins in this acute situation (39) and found no difference between HI and PI. The study was not double blind, nor randomized, the treatment schedule varied from one patient to another and ketoacids were not measured. Clark et al. (40) compared ketoacid and lipid levels in healthy volunteers during infusion of intralipid and HI or PI. They found lower B-hydroxybutyrate levels during HI. From these data it is obvious that the question wether HI is more effective than PI in the treatment of diabetic ketoacidosis is still open.

Immunogenicity of HI.

The purity of insulin preparations has been greatly improved during the last decade. Before chromatographical purification, insulin vials contained in addition to insulin also considerable amounts of proinsulin, insulin degradation products, glucagon, pancreatic polypeptide and other proteins that induce production of Ig-G antibodies. After introduction of chromatography as the endstep of the purification process, these impurities have been reduced to less than a few parts per million. Another cause of insulin antibody formation is the fact that HI differs in 3 aminoacids from beef insulin and in 1 aminoacid from PI (table 1).

Table 1. The difference of aminoacid sequence of insulin from 3 species

Species	A-chain A8	A-chain A10	B-chain B30
Man	Threonine	Isoleucine	Threonine
Pig	Threonine	Isoleucine	Alanine
Cow	Alanine	Valine	Alanine

Several studies showed marked reduction of insulin antibodies and antibodies to contaminating peptides after changing from conventionally purified beef/porcine insulin to chromatographically purified PI (41,42,43). Higher purified beef insulin also proved to be more immunogenic than highly purified PI (37). Since PI is different from HI, it should be more immunogenic in man. The expectation was that patients exclusively treated with HI would not develop insulin antibodies. However this appeared not to be the case.

Indeed it has been reported, that, compared to patients treated with PI, insulin antibodies arise in fewer newly diagnosed diabetic patients and in lower titers, when they are treated with HI (44,45). Other investigators found insulin antibodies in the same percentage of type I diabetic patients who had been treated from diagnosis on with HI or PI, but the titers were lower in the patients treated with HI (46). In an Italian multicentre study no difference in insulin antibody titers was found after 6 months of treatment (47). So the immunogenicity of HI may be lower than that of PI, but insulin antibodies still arise in patients exclusively treated with HI.

In patients who were treated with mixed beef/pork insulin containing less than 50 parts per million of proinsulin, indicating that this insulin is not highly purified, insulin antibodies decreased during a follow-up period of 6 months with treatment with HI (48). Patients who were treated during the same period with purified PI (< 5 parts per million proinsulin) showed a decrease of insulin antibodies both on continuation of purified porcine as well on transfer to HI. This decrease was not faster

nor greater during HI (48). Several investigators (24,37,38) found no change in insulin antibodies in double blind cross-over studies after 4 months of HI in patients who were previously treated with PI. From these studies it is not clear whether HI is less immunogenic than PI after more than 6 months in patients previously treated with PI, but none of the 3 studies showed a decrease of antibodies within 4 months.

So, in summary, insulin antibodies may develop less frequently in newly diagnosed diabetic patients treated with HI, compared to patients treated with animal insulin. In patients previously treated with PI, insulin antibody levels do not decrease within 4 to 6 months.

The insulin antibodies mentioned above are of the Ig-G type. Ig-E antibodies may also be present. These can induce local or generalised allergic reactions to insulin (49). In a group of patients treated with monocomponent pork or mixed beef/pork insulin, Ig-E antibody levels did not differ significantly from non-diabetic subjects, whereas patients treated with conventionally purified insulins and patients with insulin allergy had significant higher titers of anti-insulin Ig-E (50). Since HI is also highly purified, Ig-E antibodies should not arise more frequently in diabetic patients using HI than in non diabetic subjects. However, two cases of generalised allergic reactions to HI have been reported. These patients were treated previously with mixed beef/pork insulin without allergic reactions (51). Several cases were reported in which allergic reactions to animal insulin disappeared after transfer to HI (50,51,52, 53). In most of these cases it took some time before the skin reaction, which was still present after the change from animal to HI, disappeared. So far, no allergic reaction to insulin has been reported in a patient who was treated exclusively with HI.

Clinical importance of insulin antibodies.

Berson and Yalow reported in 1964 that very high concentrations of Ig-G insulin antibodies can be responsible for insulin resistance, in which the daily insulin need exceeds 200 units (54). After the introduction of chromatographically purified insulin this resistance has become extremely rare. Lower titers of insulin antibodies still arise, and the insulin

dose is possibly influenced by these low titers (55,56). Vaughan et al. (57) found that after withdrawal of insulin in patients with insulin antibodies higher levels of free insulin and lower levels of glucose were maintained for at least 12 hours as compared to patients without insulin antibodies. This buffering effect of insulin antibodies on insulin levels was also found during treatment with insulin (55,58). In a study by Bistritzer et al., however, insulin antibody binding capacity appeared to be negatively correlated with diabetic control (59). Gonen et al. found no correlation between insulin antibodies and metabolic control in 100 diabetic patients (60). So the influence of insulin antibodies on metabolic control is not clear.

The recovery from hypoglycaemias in patients with insulin antibodies is slower than in patients without antibodies, due to higher concentrations of free insulin after the hypoglycaemia (61,62). In a study using the glucose clamp technique serum free insulin concentration was not clearly influenced by insulin antibodies, but a high binding capacity of insulin antibodies was associated with a longer half live of free insulin (63). This implies that insulin levels could be inappropriately high between meals. Ludvigsson showed that newly diagnosed type I diabetic children, who develop insulin antibodies during treatment, have a shorter remission period and a higher insulin requirement than children without insulin antibodies (64). Di Mario et al. are the only investigators who suggest that immunecomplexes related to insulin antibodies are more frequent in patients with microangiopathy (65). If confirmed this observation it would mean that insulin antibodies could play a role in the development of long term diabetic complications.

In conclusion, insulin antibodies might have negative effects on the glucose regulation and possibly influence the development of long term complications in diabetic patients.

Ig-G insulin antibody assays.

Insulin antibody assays in current use can be divided in three main categories: solid phase assays, liquid phase assays using one concentration

of insulin (one point assays) and liquid phase equilibrium binding assays.

Christiansen developed a method in which the Ig-G antibodies are first precipitated, in the presence of an excess of radiolabelled insulin, on an anti-Ig-G containing agarose gel and than separated by rocket immuno-electrophoresis (66). This solid phase assay is widely used, also in most of the studies on immunogenicity of semisynthetic HI. The most important criticism on this method is that it does not take into account the dissociation between Ig-G and insulin that takes place during the electrophoresis. A second, less frequently used, method for the determination of anti insulin Ig-G is the ELISA-assay (67). Most other assays are performed with the insulin antibodies in solution. Since the Ig-G insulin complexes do not precipitate, a separation from free insulin is necessary. This separation technique of bound and free insulin can be performed by several methods: dextran coated charcoal, cellulose, gel filtration, ultracentrifugation, precipitation with salt, ethanol, polyethyleneglycol (PEG), protein A or second anti Ig-G antibody. The most widely used methods nowadays are PEG precipitation (68,69) and separation by a second antibody (70).

The first and most simple method to quantitate insulin binding to Ig-G is to add a fixed concentration of radiolabelled insulin to plasma and count the amount of radioactivity that is precipitated after separation of bound and free insulin. The insulin binding capacity can than be expressed as percentage bound or amount of insulin bound. This assay method is influenced by insulin that is present in the patients plasma. Therefore Reeves and Kelly (70) first separated insulin and anti-insulin antibody in patients plasma by dissociating the insulin-antibody complex at low pH and then absorbing free insulin to dextran coated charcoal. The determination of the "binding capacity" is than performed in this insulin free plasma by adding a fixed amount of a radioactive labelled insulin followed by precipitation of bound insulin. The radioactivity is then counted in the pellet and the amount of bound insulin can be calculated. Since binding of a ligand is influenced by binding capacity and binding affinity, it is clear that the total process of binding cannot be described completely by a single parameter. If a different insulin concentration is used in the assay of Reeves and Kelly (70) mentioned above a different

binding capacity will be the result.

Berson and Yalow (71) demonstrated in 1959 that insulin binding consists of an univalent insulin reacting with two distinctly different orders of antibody binding sites. This implicates that insulin antibodies can be described by four parameters: two binding capacities and two binding affinities. From their experimental work it became obvious that insulin antibodies consist of a low capacity, high affinity binding site and a high capacity, low affinity binding site. Berson and Yalow separated bound from free insulin by paper chromatography and they did not separate Ig-G from insulin in patient plasma. In more recent studies acidified dextran coated charcoal, as described above, was used to separate Ig-G and insulin. PEG was used to separate bound from free insulin (68,69). To describe the four parameters of insulin antibodies a vast range of insulin concentrations must be tested for amount of binding. This makes the so called equilibrium binding assays far more laborious than the one point assays. Apart from this, Reeves argues that there are probably not two but hundreds of different binding sites for insulin in insulin antibodies (72). This might be true, but the work of Berson and Yalow proved that the experiental binding results can be explained very well by a model of two binding sites and a model in which 3 binding sites were assumed did not describe the antibodies better.

A problem that influences all insulin antibody assays is non specific binding of insulin (NSB). For the calculation of specific binding, NSB has to be subtracted from total binding. Many factors of the assay method influence NSB: removal of insulin from plasma, method of iodination of insulin, incubation pH, buffer, separation method, etc. If the NSB would also vary between patients the calculation of the binding capacity is wrong, since a part of the specific binding is over- or underestimated as NSB. Several methods were introduced to make this NSB as low as possible: for instance a second antibody and a second tracer to correct for free in bound inclusion (70). It is believed by most authors that NSB is only an artefact of the assay, but it is unclear so far if it is influenced by factors of the individual plasmas. If this would be the case, the results of the assay for plasmas containing the same kind and amount of insulin antibodies, would differ between plasmas if they are not corrected for NSB for each plasma individually.

Summary and aim of the studies described in this thesis.

Two production processes are available to manufacture HI on a commercial scale. The blood glucose lowering effects of HI and PI are identical after intravenous adinistration. The absorption of subcutaneously injected HI might be slightly faster than the absorption of PI. In clinical studies small differences of home monitored blood glucoses were found between PI and HI treated groups. In newly diagnosed type I diabetic patients as well as in patients previously treated with animal insulins, HI might be less immunogenic than PI. All the above mentioned differences between HI and PI are reported by some investigators, but not confirmed by others. It is not clear whether HI is more effective than PI in the treatment of diabetic ketoacidosis.

Our aim was to investigate the efficacy and immunogenicity of HI in comparison with PI. To study the efficacy we chose to study 3 groups of patients: newly diagnosed type I diabetic patients, diabetic patients previously treated with animal insulins and patients with diabetic ketoacidosis. Glucose regulation in the first two groups was assessed by several methods, including home blood glucose estimation and HbA1 determination. To investigate the potency of HI in patients treated with animal insulins before, a group of patients was selected who injected insulin once a day. By keeping the insulin dose as constant as possible in this group of patients, a different behaviour of subcutaneously injected HI as compared with PI should become obvious in a double blind cross-over study. The study in patients with a diabetic ketoacidosis was designed as a prospective double blind study in which the treatment was identical, apart from the double blind adinistration of HI or PI.

To study the immunogenicity of HI we first developed an insulin antibody assay that describes the insulin antibody by two binding sites and that corrects for the non-specific-binding for each plasma individually. Using this assay insulin antibodies were investigated in newly diagnosed type I diabetic patients and in patients previously treated with animal insulins, comparing the immunogenicity of HI and PI treatment.

In chapter II the insulin antibody assay is described. The method used resembles most the one described by Goldmann and coworkers (68). To analyse the data we used a computerprogram describing not only specific but also non specific binding per individual plasma. From the study described in chapter IV inter- and intrapatient variance of NSB are compared to evaluate the dependence of NSB on individual plasmas.

To study the efficacy, immunogenicity and influence on residual B-cell function of HI, we compared it with PI in the treatment of newly diagnosed insulin dependent diabetic patients. In chapter III a study is described that tries to answer these questions. A randomized double blind study was designed in which patients received HI or PI and in which normoglycaemia was the aim of the treatment. By comparing insulin dose and metabolic control, efficacy of HI and PI could be investigated. The above mentioned improved insulin binding antibody assay was used to investigate the immunogenicity of the two insulins. Residual B-cell function was assessed by a meal test. This stimulation was choosen because it is more physiological than estimation of B-cell secretion after injection with glucagon.

A study on efficacy and immunogenicity of HI and PI in diabetic patients previously treated with animal insulins, is described in chapter IV. All patients were on a once a day insulin regime. The study has a double blind, randomized, cross-over design, with a run-in period. During the blind periods insulin dose was adjusted as little as possible. This constant dose in patients treated with insulin once a day, provides a better opportunity to compare the efficacy of HI and PI than the use of a free insulin dose. To assess efficacy and immunogenicity the same methods were used as in chapter II.

A study on potency of HI and PI in the treatment of diabetic ketoacidosis is described in chapter V. This study was also prospective and double blind. The treatment regime consisted of a fixed protocol of fluid, electrolyte and insulin supplements. During treatment glucose, pH, electrolytes, bicarbonate, base excess and ketoacids were measured every hour. This design, in which the treatment protocol was the same apart from the insulin species use , makes a good comparison of HI and PI possible.

After these chapters a summary of the introduction and the four studies in the English and Dutch language is provided.

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CHAPTER 2

NON-SPECIFIC BINDING OF INSULIN IN AN EQUILIBRIUM BINDING ASSAY OF INSULIN ANTIBODIES.

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Abstract.

In liquid phase assays for insulin binding antibodies (IBA), total binding of insulin is composed of specific and non specific binding (NSB). Usually NSB is determined in serum of healthy individuals and then subtracted from total binding of IBA positive serum to obtain specific binding. This method does not take into account that NSB might vary from plasma to plasma. This possibility was investigated by means of a computerised non-linear curve fitting routine for the evaluation of measurement results of an (equilibrium) binding assay for IBA, which yields estimates of NSB for each plasma individually. From each of 19 insulin treated diabetic patients, 4 blood samples, taken at different points in time, were available for IBA and NSB measurement. It was found that between patient variance of NSB exceeded within patient variance ($p < 0.01$) and, in a number of instances, within patient variance was greater than experimental variance. Our results indicate that it is advisable to use methods for IBA evaluation that take these NSB variations into account.

Introduction.

Various methods have been described for the estimation of specific binding of insulin to insulin binding antibodies (IBA) appearing in the serum of insulin treated diabetic patients (1,2,3,4,5). In all these methods some correction is applied for non-specific binding (NSB) which is estimated from the percentage of tracer insulin that is apparently bound by the serum of non insulin treated individuals. It has been assumed that NSB is purely an assay artefact, reflecting only a certain proportion of free insulin that is "misclassified" as bound through the imperfection of the free/bound separation step (3,5), and thus is only dependent on assay conditions. In this view it is justified to estimate NSB from any serum that does not contain IBA.

Thus the possibility that "free in bound" inclusion may depend on individual physicochemical plasma characteristics, is ignored. Furthermore, a plasma may contain very low affinity binding sites that cannot be saturated, but nevertheless contribute to total binding, and, if not correct-

ed for, affect the estimate of specific binding. In either case, estimates of specific binding will be less reliable when using one single NSB estimate from non IBA containing plasma for correction.
In the present study, individual estimates of NSB for different IBA containing plasmas were made in order to investigate NSB variability between plasmas from the same patient and between different patients.

Materials and Methods.

Patients and plasma collection.

Serum samples of 19 IBA positive diabetic patients were used for the study. In each subject 4 blood samples were collected, each sample with an interval of 3 months. Samples were arbitrarily considered as IBA positive if the percentage of insulin bound in the tube with the lowest insulin concentration was at least twice the percentage of insulin bound in the tube with the highest concentration of added insulin (see below). 69 Plasmas were IBA positive and 7 were negative. Each patient had at least 2 IBA positive plasmas. Of the IBA positive patients 10 were females and 9 were males, mean age was 48.3 years (range 17-80), they were on insulin therapy for a mean of 5.8 years (range 0.5-31 years). Plasma was immediately separated and stored at -20 °C. during 3 to 12 months. All plasma samples of an individual patient were analysed within one assay run. Four runs were performed.

Insulin binding antibody assay.

Insulin free IBA containing patient plasma was prepared by vortex mixing of 0.5 ml plasma with 0.1 ml 0.1 N HCL and 0.2 ml dextran coated charcoal for 5 minutes. After 5 minutes of incubation 0.1 ml 0.1 N NaOH plus 0.1 ml Veronal Human Serum Albumin (HSA) was added, followed by centrifugation at 2000 g for 10 minutes. The supernatant, containing insulin free IBA, was decanted and used for the further procedure.
For the determination of insulin binding 50 µl of supernatant, containing 25 µl insulin free plasma, was incubated during 3 days at 4°C. with

50 µl of ^{125}I-insulin (which is equivalent to 40-70 pg), and 50 µl standard insulin solution containing 0, 50, 100, 200, 500, 1000, 2000, 5000, 10.000, 20.000, 50.000, 100.000 and 200.000 pg of porcine insulin (each in duplicate), 100 µl gammaglobulin solution (10 g/ l) and Veronal-HSA up to 500 µl. After these 3 days 500 µl polyethyleneglycol (PEG) 25% (4°C.) was added and the tube was mixed and centrifuged at 2000 g during 10 minutes. Then the supernatant was removed and the radioactivity of the pellet counted in a gammacounter.

A computerised non linear curve fitting procedure according to Hermans (5) was applied to compute binding parameters from the insulin binding data, using a five parameter model for description of binding behaviour. In this model two classes of saturable insulin binding sites, with maximal binding capacities Cap1 and Cap2 and dissociation constants K1 and K2, were assumed. The fifth parameter corresponds to a constant ratio of non specifically bound-to-free insulin. It may represent both artefactual "free-in-bound" inclusion and actual binding by a third, non saturable class of binding sites. These types of binding are indistinguishable and mathematically equivalent and thus appear in the equation as a single parameter S . Bound insulin concentration (B) is expressed in terms of the five parameters and the free insulin concentration (F), according to the following equation:

$$B = (Cap1 \times F)/(K1+F) + (Cap2 \times F)/(K2+F) + (S \times F) \qquad (1)$$

This can be considered as equivalent to models previously presented by Feldman (7) and Priore et al. (8). Bound and free insulin concentrations were calculated from the total counts added, the pellet counts, and the sum of the amounts of labelled and unlabelled insulin per tube.

Goodness of fit was expressed as a chi-square value assuming an experimental replicate coefficient of variation of 5%. The results were plotted as either a bound versus free curve or a Scatchard type plot (fig. 1A and 1B).

Calculation of NSB.

In the five parameter procedure described above, NSB appears in the form of the S parameter. S values were also calculated independently, using

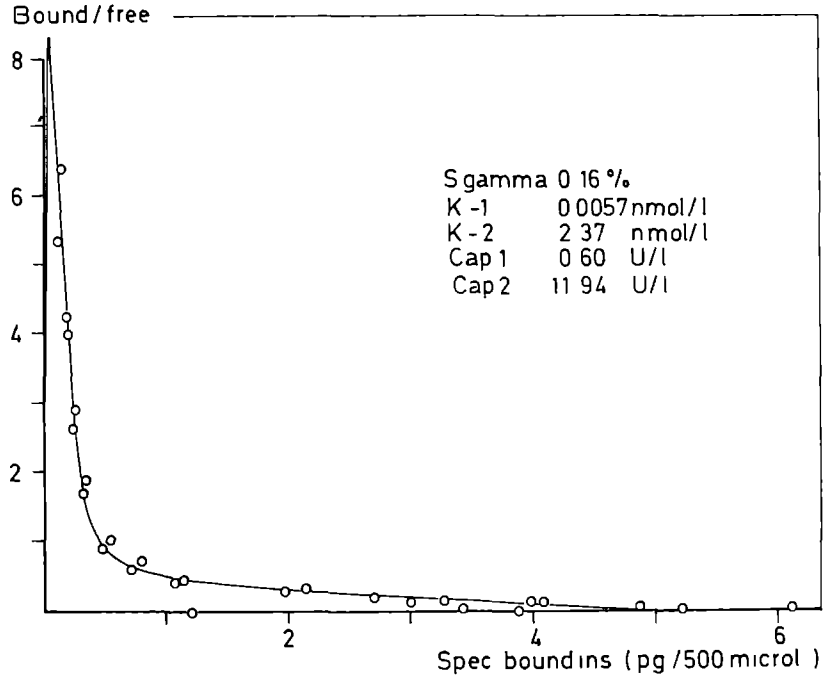

Figure 1A. Scatchard plot showing specifically bound/free versus specifically bound insulin and binding parameters in a typical analysis.

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the three highest dose points (tracer + 50.000, 100.000 and 200.000 pg/tube) by means of linear regression analysis according to the equation:

$$B = Cap1 + Cap2 + S'xF \qquad (2)$$

As can be seen, this is a particular instance of equation (1), which holds for values of the free insulin considerably exceeding K1 and K2 values. This is equivalent to full saturation of the specific binding sites.

Thus, two separate estimates of NSB, S and S' were obtained.

In plasmas of 20 healthy individuals no difference was found in percentage of bound insulin between the tube with only tracer added and the tube with 200.000 pg insulin added, indicating that there was no specific binding. For each individual plasma S was assessed by calculating the B/F ratio at three insulin concentrations (in duplicate).

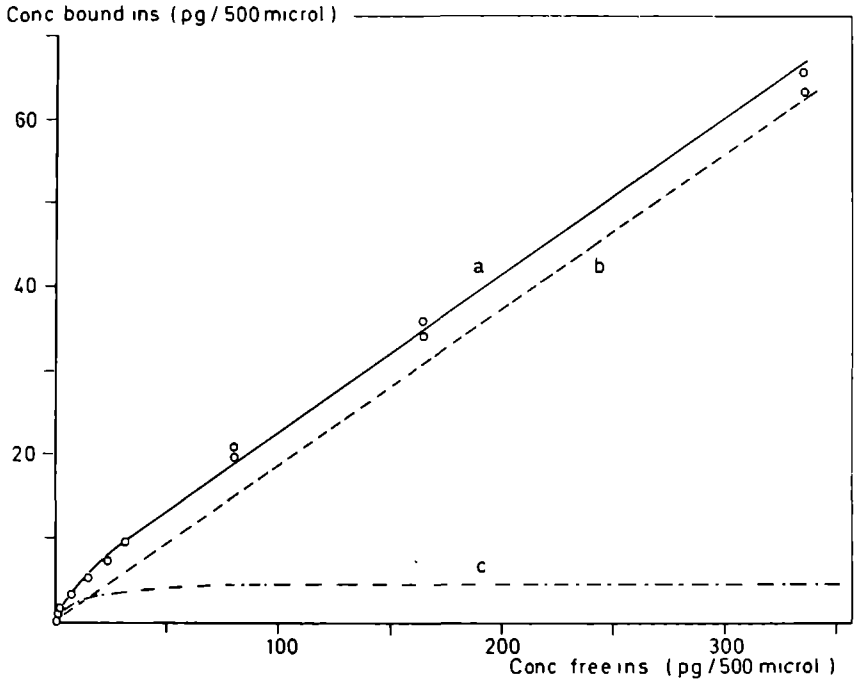

Figure 1B. Plot of bound versus free insulin. The total bound insulin (a) can be divided into a saturable specific binding (c) and a non saturable non specific binding (b)

Materials.

Veronal buffer (0.1 M; pH 8.6) containing 1.25% human serum albumin (Veronal-HSA) was used as diluting medium in all experiments. Human serum albumin (Albumine Merieux) was purchased from Rhone-Poulenc, Amstelveen, Holland. Dextran coated charcoal was prepared according to Dixons method (9). Norit GSX was obtained from Hopkins and Williams, Chadwell, England and dextran T70 from Pharmacia Upsala, Sweden. Purified porcine insulin (Novo, Bagsvaerd Denmark) was iodinated with ^{125}I by the chloramine T-method described by Hunter and Greenwood (10). The tracer was purified by chromatography on Sephadex G25 and G75 columns. Polyethyleneglycol (PEG) was manufactured by Merck-Schuchardt, Munich, F.R.G. and the gammaglobulin was obtained from Boehringwerke, Marburg an der Lahn, F.R.G.

Further calculations and statistics .

Of each patient, four plasma samples, which were collected over a 9 months period, were assayed within one run. In order to estimate within-run experimental S variance, one plasma was assayed five times. One way analysis of variance was applied to S values from each assay run for comparison of between-patient and within-patient variance, as well as for testing within-patient variance versus experimental within-run variance. Correlation between S and S' was calculated according to Pearson.

Results.

In a preliminary experiment in 20 healthy volunteers the intra individual variance exceeded the duplicate variance ($p < 0.001$).
Figure 1 shows a typical example of a binding analysis. In the second figure the plot of bound versus free insulin is shown. The curve can be resolved into non specific binding represented by a straight line trough the origin, running parallel to the upper part of the fitted line, indi-

Table 1. Intra-assay variation of the binding parameters of an equilibrium binding assay of insulin binding antibodies (N=5).

Parameter	S	K1	Cap1	K2	Cap2
Units	-	mmol/l	U/l	mmol/l	U/l
Mean	16.3	0.00857	0.7609	2.63	12.647
Standard deviation	1.23	0.00335	0.2481	0.47	1.646
Coefficient of variation (%)	7.4	39.2	32.6	18.0	13.0

K1 = dissociation constant of the high affinity antibodies
K2 = dissociation constant of the low affinity antibodies
Cap1 = maximal binding capacity of the high affinity antibodies
Cap2 = maximal binding capacity of the low affinity antibodies
S = B/F rat ∩ non specifically bound

Table 2. Analysis of inter- versus intra-patient variance and intra-patient versus intra-assay variance of non specific binding of insulin in an equilibrium binding assay of insulin binding antibodies in plasmas of 19 IBA positive patients.

Run number	Number of patients	Mean NSB	AP SD	RP SD	F-test RP versus AP	F-test AP versus AA
1	5	13.14	0.75	2.22	$p < 0.01$	NS
2	7	22.56	2.58	19.62	$p < 0.01$	NS
3	5	15.79	3.23	12.94	$p < 0.01$	$p < 0.05$
4	2	15.42	0.75	12.00	$p < 0.01$	NS

AP-SD = Intra-patient standard deviation
RP-SD = Inter-patient standard deviation
AA-SD = Intra-assay standard deviation: this was 1.23 and was analysed in run 4 (table I).

Table 3. Analysis of inter- versus intra-patient variance and intra-patient versus intra-assay variance of non specific binding of insulin in a linear regression analysis of bound versus free insulin in 19 IBA positive patients.

Run number	Number of patients	Mean NSB	AP SD	RP SD	F-test RP versus AP	F-test AP versus AA
1	5	13.1	0.73	1.77	$p < 0.01$	NS
2	7	22.6	2.46	16.85	$p < 0.01$	NS
3	5	18.3	4.26	16.38	$p < 0.01$	$p < 0.05$
4	2	16.4	1.02	13.16	$p < 0.01$	NS

AP-SD = Intra-patient standard deviation
RP-SD = Inter-patient standard deviation
AA-SD = Intra-assay standard deviation

cating saturation of specific binding, and a composite specific binding curve obtained by subtracting non specific binding from the fitted line.
In each of the 4 runs of the assay inter-patient variance of the S calculated by the 5 parameter model exceeded intra-patient variance ($p < 0.01$). In one of the four runs intra-patient variance exceeded the intra-assay variance ($p < 0.05$) (tables 1 and 2). Calculated by linear regression of the highest points of the bound versus free insulin plot inter-patient variance of the S exceeded intra-patient variance in each of the 4 runs of the assay ($p < 0.01$) (table 3).
There was a good correlation between the NSB calculated by the 5 parameter model and values calculated by linear regression on the upper 6 pairs of bound and free insulin. The correlation coefficient was high in each of the 4 runs of the assay separately as well as in the 4 runs together (table 4). The regression coefficient shows a mean overestimation of NSB in the linear regression model versus the 5 parameter model, suggesting that specific binding was not completely saturated at the dose of 50.000 or more pg of insulin.
There was no correlation between the S and maximal binding capacity of the low affinity antibodies (r=+0.21, N.S., N=69) nor between the S and the dissociation constant of the low affinity antibodies in the 5 parameter model (r=+0.07, N.S., N=69). The possible influence of duration of storage was studied by comparing NSB in the 5 model parameter in each

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Table 4. Correlation and regression analysis of NSB values determined by the 5 parameter- and linear models.

Assay run number	1	2	3	4	1+2+3+4
Corr. coeff.	0.798	0.977	0.729	0.966	0.900
Regression coeff. (b)	0.815	0.851	1.018	1.194	0.803
Constant factor (a)	0.023	0.034	0.168	-0.014	0.039
Number of observations	18	27	16	8	69
P value	<0.001	<0.001	<0.01	<0.001	<0.001

(NSB by 5 parameter model) = a + b (NSB by linear regression).

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patient in samples obtained with an interval of 6 to 9 months. There were no differences detectable in NSB between first (17.4±7.6%; mean±SD) and last (17.9±7.1%) frozen plasma (N.S., paired Wilcoxon two sample test).

Discussion.

Insulin binding antibodies can be divided into two groups, one characterised by a low capacity and high affinity and the other by a high capacity and low affinity (1,3,4). In an equilibrium binding assay the Scatchard equation is used to calculate these parameters (11). The specific binding of IBA to insulin can be found after correction of the total binding for NSB. We added the non-specifically bound fraction to the specifically bound versus free insulin equation. Thus it was possible to calculate the non-specifically bound fraction of insulin for each plasma individually. There was no significant correlation between non-specific binding and the binding parameters (maximal binding capacity and dissociation constant) of the low affinity IBA. This finding renders a poor discrimination between NSB and low affinity binding site highly unlikely.

However, the patient dependence of non specific binding could be an artefact of the complexity of the 5 parameter binding model. Therefore we calculated NSB also by a simple linear regression of the 6 highest points in the bound versus free insulin plot. Using this model, inter-patient variance was in each run of the assay also higher than intra-patient variance. There was a good correlation between the NSB calculated with the 5 parameter model and the linear regression model. The regression line shows that there is a mean overestimation of NSB in the linear regression versus the 5 parameter model. This can indicate that even at high concentrations of insulin, the insulin antibodies are not always completely saturated.

Usually NSB is calculated from IBA negative plasma (3,5). Such a practice implicitly assumes that NSB is an artefact of the assay and not influenced by properties of the individual plasma, which seems comprehensible since NSB is influenced by factors in the assay such as: whether or not the insulin is separated from IBA by the acid charcoal method, properties of the insulin tracer, the coprecipitated volume in the pellet,

concentrations of added materials as albumin and gammaglobulin.
Using the method described above we showed the variability of NSB to be larger between than within patients. In one of the four assay runs within patient variability even exceeded experimental variability. This was also the case in 20 healthy individuals. These facts show that NSB of insulin in an IBA equilibrium binding assay depends on the properties of plasma of the individual subject and possibly it even varies within patients during time.
If NSB is under- or overestimated it has considerable consequences for the low affinity binding site. Overestimation results in negative binding in the Scatchard analysis and underestimation in a too high maximal binding capacity of the two binding sites. The problem is reported by Dwenger et al. (12) who advise to calculate only the characteristics of the high affinity binding site. When binding of insulin to IBA is estimated with only one concentration of insulin (5) this problem is not noticed but the NSB influences the result anyway.
In conclusion, non specific binding of insulin in insulin binding antibody assays varies between patients and may even vary within one patient. Therefore it is advisable to take individual NSB into account, either by means of a complete parameter fit of binding data that distinguishes between specific and non specific binding, or by making a separate estimate of NSB at saturition concentrations of insulin.

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CHAPTER 3

A COMPARISON OF PORCINE AND HUMAN INSULIN IN NEWLY DIAGNOSED TYPE I DIABETIC PATIENTS: POTENCY, IMMUNOGENICITY AND THE EFFECT ON RESIDUAL B-CELL FUNCTION.

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Abstract.

The efficacy, immunogenicity and influence on residual B-cell function of semisynthetic human (HI) and monocomponent porcine insulin (PI) was compared in a double blind, randomized study in 20 newly diagnosed type I diabetic patients. 11 Patients were treated with PI and 9 with HI. Data were analyzed during a treatment period of 18 months. 7 Patients on PI and 3 on HI completed this treatment period. Using two injections a day of Actrapid and Monotard, the aim was to reach normoglycaemia. Every three months comparisons were made of HbA1, home monitored blood glucose measurements (5 times a day) and insulin binding antibodies and a meal test was performed to assess residual B-cell function. Initially HbA1 was 12.6 ± 2.2% in the PI group and 14.7 ± 1.2% in the HI group ($p < 0.05$). After 3 months of treatment this difference had disappeared and the mean HbA1 was 8.6%. The mean diurnal blood glucose concentration in both groups was not significantly different throughout the study. Also no differences could be demonstrated in blood glucose concentration at each time point separately with two exceptions: at 6 months the mean 12 a.m. blood glucose concentration and at 15 months the mean 7 a.m. blood glucose concentration were significantly higher during PI. The mean variance of glucose concentrations between days was lower during HI ($p < 0.05$). The mean of the total daily insulin dose was the same in both groups during the study, but at 12 and 18 months fewer patients on PI were treated with Monotard insulin in the afternoon. Insulin antibody characteristics, estimated with an equilibrium binding assay, did not differ between HI and PI treated patients. Residual B-cell function assessed by measurements of free insulin, proinsulin and C-peptide after a test meal, was similar in both groups at any time. It is concluded, that in newly diagnosed type I diabetic patients HI is probably as effective and as immunogenic as PI. The residual B-cell function is not influenced in a different way by HI and PI.

Introduction.

The last few years human insulin (HI) has become available for the treatment of patients with diabetes mellitus. The introduction of the treatment with HI might change the state of two clinical aspects in the treatment of insulin dependent diabetes. The first one is the metabolic control during treatment with human insulin, compared to animal insulin. In several studies the absorption of subcutaneously injected HI proved to be faster than the absorption of porcine insulin (PI) (1,2). However, no important differences in metabolic control between the treatment with the two insulin species were found in clinical studies (3,4,5,6,7).

The second aspect is the immunogenicity. During the treatment with highly purified PI, that differs in aminoacids sequence from human insulin in only one aminoacid and therefore possesses a low immunogenicity, insulin binding antibodies (IBA) still arise (8,9,10,11). Although the clinical importance of IBA is not completely understood, they are incriminated to play a role in insulin allergy (12), insulin resistance (13) and early decrease or exhaustion of B-cell function in the early phase after the diagnosis of insulin dependent diabetes mellitus (14). Since the aminoacids sequence of synthetic human insulin is the same as endogenous insulin in man, it should not be immunogenic at all. However, in several studies reported so far, IBA developed in patients treated with HI, but in a smaller number of patients and in lower concentrations than in patients treated with PI (8,9).

Several assays are used to measure IBA. The results from these assays are totally incomparable since the methods used are quite different. One problem all the methods have in common is the measurement of non specific binding (NSB) of insulin in the assay. To quantitate the specific binding of a given concentration of insulin, this NSB has to be subtracted from total binding. Since it is assumed that NSB is an artefact, that depends on assay methods only, it is usually calculated from binding of insulin in plasma of healthy individuals (15,16,17). To overcome the possible influence of individual plasma factors on NSB we developed an equilibrium binding assay that characterises IBA by two binding sites, each with its own affinity constant and capacity, and by a NSB for each individual plasma.

We performed a study in newly diagnosed insulin dependent diabetic patients in which the efficacy, immunogenicity and effect on residual B-cell function of monocomponent PI and semisynthetic HI were compared. The imunogenicity of the two insulins was assessed by the above mentioned IBA assay. Residual B-cell function was measured by a meal test.

Patients, materials and methods.

Design of the study.

Newly diagnosed type I diabetic patients were randomly assigned to double blinded HI or PI treatment. After diagnosis or after initial treatment in the event of ketoacidosis all were hospitalised for an education course of 1 week, in which the theoretical knowlegde about insulin dependent diabetes, insulin treatment and diabetic complications was tought. Patients were also instructed to inject insulin deep subcutaneously in the thigh or in the abdominal wall with a skin fold procedure (18), to perform blood glucose measurements at home with Haemoglukotest 20-800 R strips and to adjust their insulin dose using these home monitored blood glucose concentrations. The aim of the treatment was to achieve normoglycaemia. For this purpose all patients used a combination of Monotard and Actrapid insulin twice daily. Dependent on the blood sugar regulation this schedule could be simplified. After discharge from the hospital the patients visited the out-patient clinic once every one to six weeks. Before every visit and at least once every three weeks between the visits blood glucose measurements at home were performed at 8 a.m., 12 a.m., 5 p.m., 10 p.m. and 3 a.m. A 24 hours telephone service was available for problems between the visits. Every three months a meal test was performed to assess the B-cell function of the pancreas. For this purpose the patients came to the out-patient clinic in a fasting state. Before, 30, 60 and 120 minutes after their breakfast blood was collected for the estimation of glucose, C-peptide, insulin and proinsulin concentrations in the plasma. The breakfast consisted of 60 grams of carbohydrate as bread plus a glass of milk and remained the same throughout the study. HbA1, creatinine and lipid levels were determined every three months and IBA every 6 months.

Patients.

20 Patients entered the study. They all had a blood glucose level above 14 mmol/l and HbA1 above 10.0% at diagnosis. The history of weight loss, polyuria and other physical symptoms was shorter than 2 months and all had ketonuria at diagnosis. 11 Patients were treated with PI and 9 patients with HI after randomisation. There were no differences between the two groups in bloodglucose concentration, age, percentage of ideal weight (19), distribution of sex and the presence or absence of keto-acidosis at diagnosis (table 1). HbA1 at diagnosis was significantly higher in the HI treated group (table 1). The follow-up period was 2 to 36 months. Statistical analysis was performed from 0 to 18 months only. After this period the number of patients left in the HI group was too small for further analysis.

Table 1: Data at diagnosis of the patients randomized to treatment with porcine and human insulin.

Insulin	Porcine		Human
N=	11		9
Female/male	4/7		4/5
Ketoacidosis yes/no	1/10		2/7
Age in years	31.9 ± 10.8		21.9 ± 8.7
Relative weight (%)	99.2 ± 12.6		94.2 ± 12.2
Serum glucose (mmol/l)	33.4 ± 24.1		29.0 ± 18.3
Serum glucose range	14-99		15-78
HbA1 (%)	12.6 ± 2.2	*	14.7 ± 1.2
HbA1 range	10.0-18.0		13.3-17.0

* $p<0.05$

Methods.

Plasma glucose concentrations were measured with an auto-analyser using the glucose oxidase method (20). Home blood glucose measurements were performed with Haemoglucotest 20-800 R strips (Boehringer). The strips that were used the day before the visit to the out-patient clinic, were also measured with a Reflolux meter (Boehringer) at the out-patient clinic (21). HbA1 was measured by the Quick Sep Kit after an overnight incubation of the erythrocytes in 0.9% saline solution (22). Serum creatinine was measured with a continuous flow analysis with alkaline picrate. Cholesterol analysis in serum was performed with the cholesterol oxidase PAP method (23) and triglycerides with an improved semi automated method for colorimetric determination (24). The Nakagawa method (25) was used to determine free insulin concentrations. Serum C-peptide was analysed with a radioimmuno assay (26). Proinsulin was assayed at the laboratory of NOVO Industri, Bagsvaerd, Denmark (27). The IBA assay consisted of an equilibrium binding analysis, using ^{125}I-PI as the tracer and PEG as the separation step for bound and free insulin. Concentrations of bound and free insulin were calculated for 14 concentrations of total insulin. Using these data a computer program calculated binding capacity and affinity for two binding sites as well as a non-specific binding for each individual plasma. This method is described in detail in chapter 2.

Statistical analysis and calculations.

The Wilcoxon two sample test was used to analyse the differences between HI and PI treated groups. A two way variance analysis was used to compare the mean diurnal, between days and residual variance of home sampled blood glucoses during HI and PI treatment. The meal tests were analysed using the fasting value and the area under the curve for glucose, insulin, C-peptide and proinsulin concentrations as well as the increase of these parameters above the fasting-value. Ideal weight was expressed as the percentage of the following value:

women: ideal weight = $3.265 \times \text{height}^{1.9104}$

men : ideal weight = $3.699 \times \text{height}^{1.9003}$

These formulas were obtained after loglinear regression of the weight and height tables of the Metropolian Life Insurance Company (19) ($P < 0.01$; $R = 0.99$).

The analyses were performed from 0 to 18 months. After this period 7 patients had completed the study in the PI group and 3 in the HI group. After the double blind study was ended the patients continued the insulin they had before in an open study that is still running. IBA were analysed every 6 months. Two patients in the HI group were in the blind study for periods between 15 and 18 months. Their IBA values after 18 months were analysed while being in the open study. Including these two patients, 7 patients in the PI group and 5 patients in the HI group completed 18 months of the study.

Results.

Complications.

During the study no patient suffered from a hypoglycaemia that made a medical intervention necessary, nor had any of them a period of ketoacidosis after diagnosis. No allergic reactions to insulin were observed. As could be expected no retinopathy nor clinical nephropathy was found in any of the patients, as judged by fundoscopy and proteinuria.

Blood glucose.

At diagnosis no statistically significant differences in blood glucose concentrations could be found between the two groups (Table 1). The blood glucose taken at a random time after breakfast during visits at the outpatient clinic showed a significant difference between HI and PI groups only at the first visit (Table 2). The mean home blood glucose concentration at 7 a.m., 12 a.m., 5 p.m., 10 p.m. and 3 a.m., taken at intervals of 3 months on the day before the meal test in the HI and PI groups were compared up to a total period of 18 months. Of these 30 Wilcoxon two sample tests only 2 reached a significant value. After 6 months of treatment the mean 12 a.m. blood glucose was 5.4 ± 0.8 mmol/l in the HI group

Table 2: Glucose concentration (mmol/l) at the out-patient clinic for porcine and human insulin treated patients.

Insulin		porcine	N		human	N
Months of treatment	0	7.8 ± 2.8	10	*	12.2 ± 4.2	6
	3	8.5 ± 2.2	10		9.2 ± 1.6	8
	6	9.0 ± 1.9	9		8.8 ± 1.8	8
	9	8.9 ± 2.3	7		7.4 ± 2.6	7
	12	10.4 ± 3.6	7		9.9 ± 2.2	6
	15	10.4 ± 3.6	7		8.0 ± 1.7	5
	18	11.5 ± 4.7	7		9.3 ± 2.1	3

* P<0.05

Table 3: Mean ± SD of the home blood glucose (mmol/l) at different time points of the study for porcine and human insulin treated patients.

Insulin		porcine	N	human	N
Months of	0	10.6 ± 4.7	11	11.6 ± 5.6	9
treatment	3	7.4 ± 1.5	11	7.3 ± 1.2	8
	6	8.9 ± 2.5	9	7.1 ± 1.1	8
	9	7.6 ± 1.4	7	6.6 ± 2.1	7
	12	8.2 ± 1.2	7	7.8 ± 1.9	6
	15	8.6 ± 2.0	7	6.8 ± 0.8	5
	18	9.5 ± 2.4	7	8.5 ± 2.3	3

(N=9) and 9.7 ± 3.1 mmol/l in the PI group (N=8) (P<0.05). After 15 months of treatment the mean 7 a.m. blood glucose was 7.4 ± 1.4 mmol/l in the HI group (N=5) and 10.4 ± 2.7 mmol/l in the PI group (N=7) (P<0.05). Since several patients refused to take home blood glucoses at 3 a.m. regularly, these figures were not available for all patients. For this reason the mean home blood glucose concentration was calculated from the other 4 time points. No differences were found between HI and PI groups for mean home blood glucose (Table 3).

To evaluate the variability of the home blood glucose concentrations, a two way variance analysis was performed. For this calculation the glucose concentrations at 7 a.m., 12 a.m., 5 p.m. and 10 p.m. after 3, 6, 9, 12 and 15 months of treatment were used. These values were complete for 7 patients in the PI treated group and for 5 patients in the HI treated group. For each of these patients the diurnal, between days and residual variance of the home blood glucose concentrations were calculated. The mean between days variance was significantly higher and the residual variance tended to be higher during PI (Table 4).

Table 4: Mean ± SD variance of home blood glucose at 3, 6, 9 12 and 15 months, compared between human and porcine insulin treated patients.

Insulin	porcine		human
Between days variance	15.0 ± 10.0	*	6.6 ± 2.5
Within days variance	9.6 ± 8.9		4.5 ± 3.5
Residual variance	6.1 ± 2.2	o	2.9 ± 2.1

* $p<0.05$
o $0.05<p<0.10$

HbA1.

At the start (Table 1) and at the first visit to the out-patient clinic HbA1 was higher in the HI group. At no other time significant differences were found between HI and PI treated groups (Fig. 1). During treatment the mean HbA1 was 8.6%. The upper limit of the HbA1 in healthy subjects is 7.9% in our laboratory (Mean + 2 SD).

Insulin dose.

In the PI group 5 out of 11 patients injected no insulin for 1.9 ± 1.3 (mean ± SD) months. In the HI group 3 out of 9 patients injected no

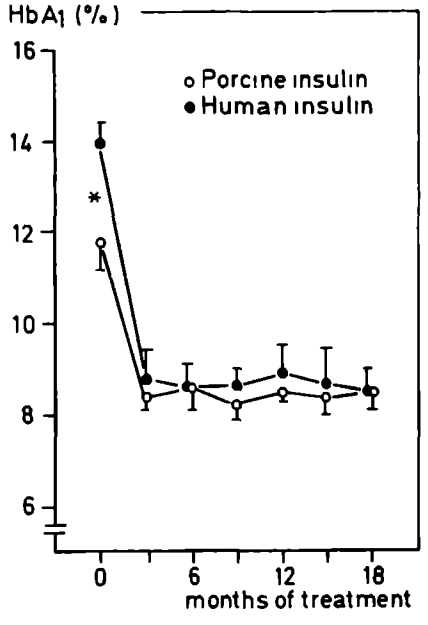

Figure 1: Mean ± SE HbA1 (%) in type I diabetic patients treated with human (•) or porcine (o) insulin. At the start the HbA1 concentration was significantly higher in the human insulin treated group (* $p<0.05$).

insulin for 8.0 ± 6.1 months. No significant differences in the percentage of patients who reached a complete remission nor in the duration of the remission were found in the HI and PI treated group.

Monotard and Actrapid doses were similar in the morning and in the afternoon for HI and PI groups, with two exceptions. At 12 months the HI group (N=6) needed in the afternoon a mean of 4.3 ± 5.1 units of Monotard, while all 7 patients of the PI group injected no Monotard insulin in the afternoon ($P<0.05$). At 18 months the HI group (N=3) injected 6.0 ± 6.2 units of Monotard and the PI group (N=7) injected no Monotard insulin at 5 p.m. ($P<0.05$).

The total insulin dose was not signifficantly different in PI and HI groups at any time point (Table 5).

Table 6: Mean ± SD fasting creatinine (μmol/l), cholesterol (mmol/l) and triglyceride (mmol/l) levels at different time points of the study for human and porcine insulin treated patients.

		Creatinine		Cholesterol		Triglyceride		N	
Insulin		Porcine	Human	Porcine	Human	Porcine	Human	Por.	Hu.
Months of	0	75.2± 8.7	76.1±14.4	5.3±0.9	4.6±1.4	1.44±0.73	1.40±0.43	11	9
treatment	3	73.8±10.7	73.6±12.9	4.7±0.8	4.4±1.0	1.22±0.57	0.89±0.56	11	8
	6	71.2±11.8	73.0± 9.7	4.9±0.4	4.4±1.2	1.16±0.55	1.03±0.60	9	8
	9	70.6±11.5	75.0±13.2	4.9±0.5	4.8±1.3	0.89±0.17	1.07±0.62	7	7
	12	68.0± 7.9	75.7±10.9	4.9±0.5	4.4±1.2	0.99±0.25	0.94±0.47	7	6
	15	72.3±12.9	75.0±11.3	4.8±0.3	4.5±1.3	0.97±0.29	1.04±0.49	7	5
	18	68.9±11.1	74.3±11.0	4.7±0.4	* 3.8±0.6	0.87±0.20	0.53±0.32	7	3

* $P<0.05$

Table 5. Mean ± SD daily insulin dose at different time points of the study for human and porcine insulin treated patients.

Insulin		Porcine	N	Human	N
Months of treatment	0	32.4 ± 16.3	11	28.9 ± 15.5	9
	3	16.2 ± 13.0	11	24.0 ± 17.5	8
	6	13.1 ± 5.9	9	21.5 ± 18.4	8
	9	18.9 ± 6.2	7	25.0 ± 20.0	7
	12	22.9 ± 9.6	7	22.0 ± 14.7	6

Creatinine, cholesterol and triglycerides.

At no time point creatinine or fasting triglyceride levels were different in HI and PI treated groups. Cholesterol levels were lower during HI after 18 months only (PI: 4.7 ± 0.4 mmol/l, N=7, and HI: 3.8 ± 0.6 mmol/l, N=3, $P<0.05$) (Table 6).

B-cell stimulation test (meal test).

The fasting levels of glucose, free insulin, C-peptide and proinsulin were not significantly different in HI and PI groups at any time point (Table 7). To evaluate the residual function of B cells of the pancreas, the area under the curve untill 2 hours after breakfast was calculated for the 4 parameters. At no time point significant differences were found between PI and HI groups for these areas under the curve (Fig. 2). To assess the reserve capacity of the B cells the area under the curve was calculated after subtraction of the fasting value for each of the parameters. Also for this reserve capacity no significant differences were found between HI and PI groups (Fig. 2).

Insulin antibodies.

Insulin antibodies were assayed after 0, 6, 12 and 18 months in 7 patients in the PI and 5 patients in the HI group as indicated in the

Table 7: Mean ± SD levels of fasting glucose (mmol/ml), C-peptide (pmol/ml) free insulin (mU/l) and proinsulin (pmol/ml) at different time points of the study for human and porcine insulin treated patients.

		Glucose		C-peptide		Insulin		Proinsulin		N	
Insulin		Porcine	Human	Porcine	Human	Porcine	Human	Porcine	Human	Por.	Hum.
months	0	8.3±2.7	10.8±3.5	0.28±0.15	0.32±0.10	12.2±4.6	9.2±3.7	0.036±0.046	0.028±0.020	11	8
of	3	7.8±1.4	8.3±1.8	0.36±0.18	0.32±0.12	10.1±4.1	12.7±4.1	0.077±0.057	0.055±0.044	11	8
treatment	6	9.1±1.9	8.7±1.7	0.41±0.19	0.34±0.20	12.1±4.6	10.5±4.3	0.084±0.051	0.057±0.032	9	8
	9	8.3±1.9	7.6±2.4	0.29±0.12	0.23±0.10	11.0±3.5	11.3±2.3	0.110±0.116	0.054±0.057	7	7
	12	10.5±3.3	9.8±2.4	0.29±0.13	0.24±0.07	10.6±2.9	11.3±1.5	0.083±0.069	0.061±0.079	7	6
	15	9.7±1.9	8.0±1.7	0.21±0.09	0.21±0.09	7.0±2.0	10.2±3.3	0.054±0.053	0.034±0.048	7	5

Normal fasting values in healthy subjects:

C-peptide 0.53 ± 0.16 pmol/l

Insulin 7.7 ± 4.8 mU/l

Proinsulin 0.024 ± 0.020 pmol/l

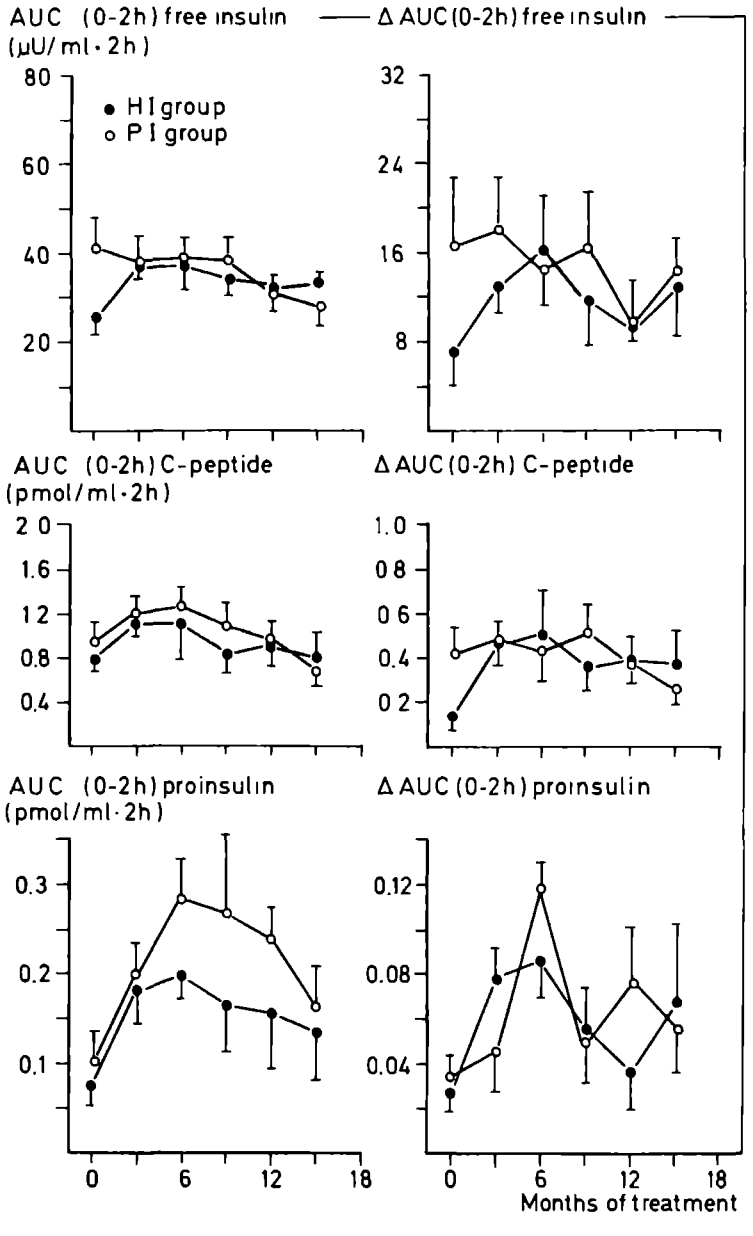

Figure 2: Mean ± SE area under the curve (AUC) of free insulin, C-peptide and proinsulin concentrations after stimulation of their production with a meal test in human and porcine insulin treated patients. The tests were performed with intervals of 3 months. In the left pannels the AUC is calculated for the concentrations and in the right pannels the AUC curve is calculated after subtraction of the premeal value (Δ AUC). No significant differences between the human (HI) and porcine (PI) insulin treated group were found.

Table 8: Mean ± S.D. insulin antibody parameters after 6, 12 and 18 months of treatment with porcine and human insulin. Included are 7 patients who were treated with porcine and 5 patients who were treated with human insulin.

Time point	6 Months		12 months		18 months	
Insulin	Porcine	Human	Porcine	Human	Porcine	Human
Antibody +/-	3/4	3/2	5/2	2/3	4/3	2/3
K1 (nmol/l x 0.001)	5.66±4.67	1.77± 1.48	14.16± 8.80	8.40± 9.18	8.56±1.08	19.97± 3.78
K2 (nmol/l x 0.1)	4.67±3.74	108.8±178.2	19.63±14.40	10.71±13.61	27.67±39.45	168.8±53.03
Cap1 (U/l x 0.01	4.20±9.43	5.91±10.59	6.77± 8.53	4.30± 8.93	2.79± 3.00	5.79± 8.05
Cap2 (U/l)	0.97±2.39	2.92± 5.76	1.30± 1.73	0.55± 1.08	1.10± 1.70	3.92± 6.17

K1: Dissociation factor of high affinity antibodies. For calculations only antibody positive figures are used.

K2: Dissociation factor of low affinity antibodies. For calculation only antibody positive figures are used.

Cap1: Capacity of high affinity antibodies. For calculation antibody positive and negative figures are used.

Cap2: Capacity of low affinity antibodies. For calculation antibody positive and negative figures are used.

methods. At the start no specific binding of insulin was found in any of the sera. No differences between the two groups were found in non specific binding at any time. In both groups there was one patient who had specific binding for insulin after 6 months, but lost this binding capacity at a later time. No significant differences were found between binding capacities or affinities for high and low affinity binding sites at any time between HI and PI treated groups. No differences were found in the ratio binders/non binders at any time point (Table 8).

Discussion.

After the introduction of new production techniques, human insulin has become available for the treatment of diabetic patients. In addition to its practical and economical benefits, human insulin might also have advantages from a clinical point of view. Treatment with this most physiological insulin species might in the first place result in a better metabolic control, compared to treatment with animal insulin species.
To study this, we treated newly diagnosed insulin dependent diabetic patients with semisynthetic HI or monocomponent PI and tried to reach the best possible metabolic control with two insulin injections a day. The total insulin dose was not significantly different in the two groups at any point of time. As far as the distribution of the insulin dose during the day is concerned, virtually no difference was found between the two groups. Many blood glucose concentrations were measured at home during the study at fixed time points during the day. Only 2 differences in home blood glucose concentrations were found during the whole study. After 6 months at 12 a.m. and after 15 months at 7 a.m. the blood glucose concentration was higher in the PI treated group. Since these are the only 2, no consistent difference in home blood glucose concentrations between the HI and PI treated groups was found. However, the between days variance of blood glucose levels was significantly higher during PI treatment. This suggests that a more stable metabolic control is possible during the treatment with HI. However, the differences between HI and PI are that small, that their clinical relevance remains doubtful.

Slight but clinically unimportant differences were found in some studies (3,4,5), while others found no differences in metabolic control (6,7) comparing HI with PI. Our opinion after consideration of the results of other and our studies, is that, compared to PI, HI has no important advantage for the treatment of insulin dependent diabetic patients.

The second possible advantage of HI would be an absence of immunogenicity. Although insulin binding antibody levels are low during treatment with monocomponent PI, they can play a role in allergy (12), and might influence insulin pharmacokinetics (28,29) and residual B-cell function (14). In 7 patients treated with PI and in 5 patients treated with HI the IBA could be analysed after 0, 6, 12 and 18 months of treatment. In contrast with results reported before (30) no IBA were found before treatment with insulin. At no time after diagnosis we found significant differences in binding parameters between PI and HI treated patients. In some other studies in newly diagnosed diabetic patients lower IBA levels were found in HI treated patients (8,9). There might be two reasons for this discrepancy. The first is the difference in the IBA assay used. Not only the technical part of the assay, but most of the principles on which it is based differ. In our assay binding capacity and affinity of 2 different binding sites are estimated with a correction for NSB in each individual plasma. In other, less laborious assays, this distinction of two binding sites and the individual correction for NSB is not made and therefore results are not comparable. Fineberg et al (9), who used an assay that also describes capacity and affinity for two binding sites, but that not corrects individually for NSB, found also no difference in IBA during treatment with HI and PI in newly diagnosed type I diabetic patients. A second possible explanation might be a type 2 error in the statistical analysis, because of the low number of patients in each group.

In a recent study it is suggested that even low titers of insulin antibodies are associated with progressive loss of B-cell function (14). When HI is less immunogenic than PI this should result in a less pronounced loss of B-cell function during HI. We tested in all patients residual B-cell function with a physiological standardised breakfast test. No differences in fasting values nor in rise of C-peptide and free insulin levels after breakfast were found, between the groups treated with HI or

PI. So, in our study, it was not possible to find a significant difference in residual B-cell function after treatment up to 18 months with PI or HI. After a mean of 6 months of treatment maximal free insulin, C-peptide and proinsulin levels were found. After 15 months proinsulin levels were decreased as compared to 6 months but were still higher than in normal individuals (27). For this high proinsulin levels there are two possible explanations. The first one is that proinsulin binds to IBA thus prolonging its presence in the blood. However, IBA are relatively constant after 6-12 months while proinsulin concentrations first increase and than decrease. Moreover, C-peptide concentrations vary paralell with proinsulin levels, indicating a relationship at the level of the B-cell. The second explanation is a high output of proinsulin by the B-cell. This hypothesis is confirmed by Heding et al (31) who found an abnormally high proinsulin/insulin ratio in type I diabetics in the first year after diagnosis.

In conclusion we found that HI in newly diagnosed type I diabetic patients is as effective and as immunogenic as PI. The residual B-cell function is not influenced in a different way by HI and PI.

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CHAPTER 4

EFFICACY AND IMMUNOGENICITY OF HUMAN AND MONOCOMPONENT PORCINE INSULIN: A RANDOMIZED DOUBLE BLIND STUDY IN DIABETIC PATIENTS PREVIOUSLY TREATED WITH INSULIN.

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Abstract.

In 32 insulin treated diabetic patients the efficacy and immunogenicity of monocomponent porcine (PI) and semisynthetic human (HI) insulin were compared in a randomized, double blind, cross-over study. After a run-in period of 3 months the patients were randomized into group I (N=19) treated with porcine insulin first and then with human insulin for periods of 3 months and group II (N=13) treated with human insulin first and porcine insulin subsequently. The insulin dose was kept as constant as possible and differed not between the two groups. During HI in group II there was a significant increase of HbA1 (11.2±1.3 to 11.6±1.4%, $p < 0.05$). The mean blood glucose at 12 a.m. during HI in group I exceeded the one during PI (15.0±6.3 (HI) and 12.7±4.6 mmol/l (PI), $p < 0.05$). For both groups together the diurnal variance of blood glucoses during PI exceeded the one during HI ($p < 0.05$). No other differences were found. Insulin antibodies were determined by an equilibrium binding assay. 19 Patients were IBA positive. During HI the low affinity binding site capacity decreased for group I and II together ($p < 0.05$) and the capacity of both binding sites decreased in group II ($p < 0.05$). We conclude that there are small differences in the efficacy of human and porcine insulin that are of no clinical importance and that after changing from porcine to human insulin, insulin antibodies decrease within 3 months.

Introduction.

The last few years human insulin (HI) has been available for the treatment of diabetic patients. It differs from porcine insulin (PI) in only one aminoacid. Human insulin can be produced by the recombinant DNA method (Biosynthetic Human Insulin) (1) and by enzymatic replacement of alanine by threonine in the B30 position of the porcine insulin molecule (Semisynthetic Human Insulin) (2).

So far no clinically important differences in efficacy have been found between highly purified porcine and human insulin (3-7). According to some studies (6,7,8) human insulin is absorbed slightly faster from subcutaneous tissue than porcine insulin, but in other studies (9,10) no difference was found.

A theoretical advantage of human insulin could be its absent or low immunogenicity in diabetic patients, although the importance of insulin antibodies from a clinical point of view is not clear, apart from rare cases of insulin resistance and insulin allergy. Since beef insulin is more immunogenic than porcine insulin (11,12), a comparison between human and porcine insulin seems the next logical step. It has been shown in newly diagnosed type I diabetic patients, treated with human insulin, that insulin antibodies still arise but in a smaller percentage of patients and in lower titers (13,14,15). In studies in patients previously treated with animal insulins, HI caused a decrease of insulin antibodies in some studies (13), but in others no change was found (16,17). The studies on immunogenicity of HI are difficult to compare because of the different assays for insulin antibodies.
We studied the efficacy and immunogenicity of HI. A double blind randomized cross-over study was designed, in which semisynthetic HI and monocomponent PI were compared. The study was performed in patients previously treated with insulin. We studied insulin binding antibodies more extensively than was done in most studies so far, and we used an assay in which non specific binding is accounted for in a new manner.

Materials and methods.

Design of the study.

In a run-in period of 3 months, the selected patients were treated with PI. During this period the treatment was adjusted, the diet was revised and altered if necessary, injection technique was inspected and education was given if necessary. Next the patients were randomized into two groups and the study was continued in a double blind way for patient and physician as far as the type of insulin was concerned. In the first group PI was continued for another 3 months and than replaced by HI during a period of 3 months (group I). In the second group the patients received first HI and then PI (group II).
The insulins used in this study were monocomponent Actrapid and Monotard insulin, both porcine and human. Only 3 patients used Actrapid insulin, 2 in group I and 1 in group II.

The insulin dose during the blind periods was essentially not changed. Only subjective hypoglycaemias and blood sugars below 3.0 mmol/l or hyperglycaemias above 20.0 mmol/l, that could not be explained by other reasons than insulin dose, caused dose adjustments. During the study all patients visited the out-patient clinic once in every 3 weeks in the morning after breakfast where all were seen by the same physician.

Patients.

43 insulin dependant diabetic patients, willing to cooperate and using insulin for at least half a year, were selected. They all injected insulin once a day (long acting with or without short acting insulin) for various reasons, for instance high age or refusal to use more than one injection. None of them had serious diabetic complications. All patients gave informed consent and the protocol of the study was approved by the ethical committee of our hospital.
37 Patients reached the end of the run in period. Six patients dropped out during the run-in period because of personal reasons or medical reasons not connected with insulin use. During the blind periods there were 5 more drop-outs: 2 for personal reasons, 1 because of a progressively increasing insulin need and 2 because of serious hypoglycaemias, both within 2 weeks after the start of HI. 32 Patients completed the study, 19 in group I and 13 in group II (figure 1).
The basal data of the patients who completed the study are shown in table 1. As to these data there were no significant differences between the two groups.

Parameters of diabetes control.

The day before each visit to the out-patient clinic the patients collected at home their 24 hours urine divided in 4 portions (8-12a.m., 12a.m.-6p.m., 6-11p.m., 11p.m.-8a.m.). Blood glucose monitoring was performed with Reflotest (Boehringer) strips at 7 a.m., 12 a.m., 5 p.m., 11 p.m. and 3 a.m. The next day these strips were measured (Reflomat, Boehringer) at the out-patient clinic (18). The urine was tested for glucose and ketone bodies by Gluketur strips (Boehringer). If the test was posi-

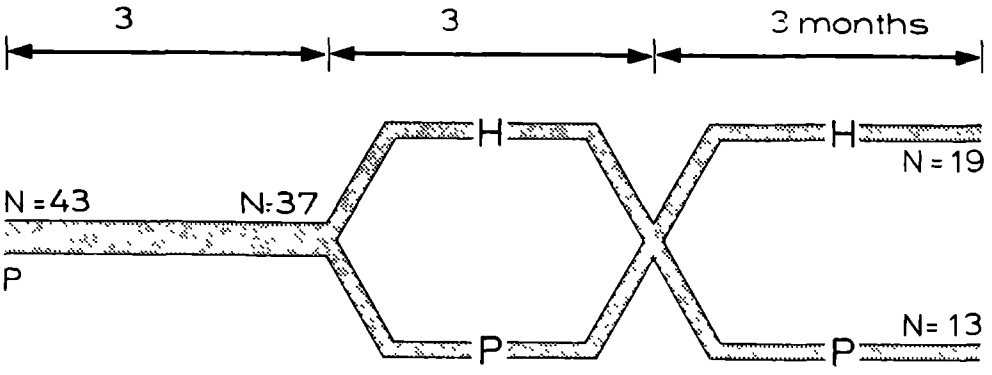

Figure 1: Design of the study and numbers of patients.
P indicates treatment with PI and H treatment with HI. For the reason why patients dropped out of the study see text.

Table 1: Data at the start of the study of the patients who completed the study.

		Total group	Group I	Group II
N		32	19	13
women/man*		16/16	11/8	5/8
type I/II sec. failure**		18/14	11/8	7/6
mean age (range)	yr	48 (18-80)	50.0±16.9	45.4±19.7
duration diab.-mellitus	yr	12 (0.5-39)	12.3±10.9	11.2±10.7
duration insulin-therapy	yr	8.2 (0.5-39)	8.2±11.1	8.2±10.5
% of ideal body weight	%		109±13	102±13
length	cm		169±8	173±11

* A chi square test was used to compare sex and type I/II diabetics. For the other parameters the student T test was applied. No significant differences were found.

** Secondary failure in type II diabetic patients means that it was necessary to transfer patients from tablets to insulin.

tive for glucose a quantitative measurement was performed. Plasma glucose, HBA1 and bodyweight were measured at each visit to the out-patient clinic. The patients were asked for the number of subjective hypoglycaemias, causing them to take carbohydrates, that they had experienced between the two visits. At the start of the study and at the end of each period of 3 months insulin antibodies were determined.

Methods.

Ideal weight was calculated by a formula obtained after loglinear regression from the tables for medium frame size of the Metropolitan Life Insurance Company (19). A good correlation was found for the line fitted in this way both for women and men ($R = 0.99$; $P < 0.01$). The formulas are as follows:

$$\text{Women} : \text{ideal weight} = 3.265 \times \text{height}^{1.9104}$$

$$\text{Men} : \text{ideal weight} = 3.699 \times \text{height}^{1.9003}$$

When the height is expressed in centimeters, the ideal weight (IW) is expressed in grams. The percentage deviation of the ideal weight (DIW) is calculated as: DIW = (weight - IW) x 100 / (IW).
Glucose in plasma and urine was measured with an autoanalyser using the glucose oxidase method (20). HbA1 was measured after an overnight incubation of the erytrocytes in 0.9% saline by the Quick-Sep Kit (21). Insulin binding antibodies (IBA) were analysed by an equilibrium binding assay, characterising the antibodies by two specific binding sites, each one having its own affinity and capacity. Insulin and IBA were separated by the method described by Dixon (22). To the insulin free plasma a ^{125}Iodine porcine insulin tracer and increasing amounts of non labelled porcine insulin were added. After an incubation of 3 days at 4°C, bound insulin was precipitated by polyethyleneglycol 25% (PEG). The pellet was than counted in a gamma counter. Using these counts pairs of bound and free insulin were calculated and than fitted in a non linear computer program by iteration using the following formula:

$$B = (Cap1 \times F)/(K1 + F) + (Cap2 \times F)/(K2 + F) + S \times F$$

B = concentration of bound insulin.
F = concentration of free insulin.
Cap1 and Cap 2 = maximal binding capacity of first and second binding site of insulin antibodies.
K1 and K2 = dissociation constant of first and second binding site of insulin antibodies.
S = ratio B/F non specifically bound.

For each plasma containing IBA these parameters were calculated. New in this assay is the assessment of non specific binding of insulin per individual plasma.
Changes of HbA1, insulin dose and IBA were tested with a paired Wilcoxon test comparing parameters at the start and the end of the periods. Unless mentioned otherwise group I and group II were analysed separately. This was done because the HI period always followed a PI period, but the PI period did not always follow a HI period (fig.1). Moreover, the groups did not comprise the same number of patients. Variability of home monitored blood glucose was analysed using a two way variance analysis. The mean of the parameters obtained at all visits during a period was calculated. The mean values of HI and PI periods were compared by a paired Wilcoxon two sample test. The results are expressed as mean ± SD. If other tests were used this is mentioned.

Results.

Insulin dose.

The mean insulin dose was the same at the start and at the end of HI and PI periods (table 2). During 88 visits to the out-patient clinic in group I the insulin dose was adjusted 12 times in a total of 7 patients of group I during PI. The mean of the dose changes was +0.2±4.6 units of insulin. During 86 visits in group I the dose was adjusted 13 times in a total of 8 patients during HI. The mean of the dose changes was +1.1±3.6 units of insulin. There was no significant difference between HI and PI periods in dose adjustment in group I. During 55 visits in group II the

dose was adjusted 5 times in a total of 3 patients during PI. The mean of the dose changes was +0.4±4.1 units of insulin. During 66 visits in group II the dose was adjusted 6 times in a total of 3 patients during HI. The mean of the dose changes was 0.0±4.4 units of insulin. There was no significant difference in dose adjustments between HI and PI periods in group II.

Table 2: Insulin dose (U/24 hours) at the end of each period.

	run-in period	period 1	period 2
Group I (N=19)	30.1±11.6 (PI)	29.7±10.4 (PI)	29.5±10.7 (HI)
Group II (N=13)	28.9± 9.7 (PI)	30.3±12.5 (HI)	30.2±12.1 (PI)

(PI) means treatment with porcine insulin.
(HI) means treatment with human insulin.

No significant differences were found between end of period 1 and 2 in both groups.

Glucosuria and ketonuria.

The mean glucosuria during 24 hours was calculated for each patient individually in HI and PI periods. As well in group I (PI period 32.8 and HI period 40.8 g/24hours) as in group II (HI period 64.5 and PI period 55.2 g/24 hours) the mean glucosuria during HI exceeded the mean glucosuria during PI. These differences however were not statistically significant. When glucosuria in the four portions of the day was analysed it appeared to be significantly higher from 12 a.m. to 6 p.m. during HI in both groups. In group I glucosuria was also higher during HI from 8 a.m. to 12 a.m. (table 3).

Table 3: Mean glucosuria (= S.D.) in grams during HI and PI, tested for the different parts of the day and for 24 hours.

	GROUP I			GROUP II		
PERIOD	HI		PI	HI		PI
8-12 am	10.4± 7.5	*	6.9± 5.9	15.9± 6.6		15.8± 9.0
12am-6pm	9.8± 9.7	*	7.3± 8.9	19.8±12.6	*	11.6± 5.1
6-10pm	9.1±10.3		8.5± 8.3	14.9± 8.7		11.2± 7.2
10pm-8am	11.5± 7.0		9.8± 7.3	13.6± 9.5		16.1±13.9
24 hours	40.8±28.8		32.8±25.0	64.5±29.6		55.2±25.6

* $p < 0.05$

Ketonuria was very infrequent. In group I ketonuria was found in 4 out of 352 urine samples during PI and 8 out of 340 urine samples during HI. In group II ketonuria was found in 1 out of 220 urine samples during PI and 0 out of 256 urine samples during HI. The differences between HI and PI periods were not statistically significant.

Blood glucose.

Each patient was asked to perform at home a blood glucose test 5 times a day. The range of the Reflometer is between 3 and 20 mmol/l. Since there were figures beyond this range, differences between HI and PI periods were analysed using a sign test. For the calculation of the median blood glucose values in the individual patients, concentrations below 3.0 mmol/l were considered to be 3.0 mmol/l and above 20.0 mmol/l were calculated as 30.0 mmol/l arbitrary. To arive at the mean value of the group the individual median values were avaraged. No significant differences in mean blood glucose values between HI and PI periods were found at 8 a.m., 5 p.m., 11 p.m. and 3 a.m. The mean glucose at 12 a.m. was 15.0±6.3 mmol/l during HI and 12.7±4, 6 mmol/l during PI in group I ($P < 0.05$). In group II no significant difference was found at this point (table 4).
To evaluate the variability of home blood glucose, a two way variance

analysis was performed for group I and II together. No differences of between days and residual variance of blood glucoses between HI and PI periods were found. The diurnal variance during PI exceeded the diurnal variance during HI significantly ($p < 0.05$).

We calculated the mean chance that the blood sugar measured during the out-patient clinic visit was higher during HI than during PI for each patient individually by comparing each blood sugar during HI with all blood sugars during PI. A chance of 0.5 indicates that there are no differences in bloodsugar between HI and PI periods. In group I a significant deviation from 0.5 was found (0.66, $p < 0.05$).

Table 4: Mean of the median home monitored blood glucoses in mmol/l for the five time points of the day during HI and PI in group I and II.

	GROUP I			GROUP II		
PERIOD	HI	PI	(N)	HI	PI	(N)
8am	10.9±5.2	9.4±3.2	(18)	13.5±6.0	13.2±6.1	(13)
12am	15.0±6.3 *	12.7±4.6	(18)	16.2±8.0	13.3±4.1	(13)
5pm	11.0±6.1	11.1±5.4	(18)	14.1±9.5	12.9±5.9	(13)
22pm	14.8±6.4	15.0±5.7	(18)	16.9±7.7	15.3±7.4	(12)
3am	13.7±5.2	11.9±3.3	(14)	12.8±3.7	14.3±6.9	(11)

* $p < 0.05$ tested with sign test.

Table 5: Mean of % HbA1 (±SD) for group I and II at the start and the end of the different periods of the study.

	run in period		period 1	period 2
	Start	End	End	End
group I (N=19)	12.1±2.1 *	11.1±1.4	11.4±1.8(P)	11.9±1.9(H)
group II (N=13)	12.2±2.0	11.2±1.3 *	11.6±1.4(H)	11.5±1.3(P)

* $p < 0.05$

The insulin species that was used is given in parentheses.

HbA1

During the first 6 weeks of the run-in period there was a significant decrease in percentage of HbA1. During HI in group II there was a small but significant rise in HbA1. No changes were found during PI or during HI in group I, nor during PI in group II (table 5).

Hypoglycaemias.

The mean frequency of subjective hypoglycaemias was less than once a week. We calculated the chance that one patient had at least one subjective hypoglycaemia between two visits to the out-patients clinic during the HI or PI period. The mean chances were not different between HI and PI in group I and II (table 6). There were also no differences in time of the day of hypoglycaemias between HI and PI.

Table 6: Chance of getting at least one subjective hypoglycaemia between two visits at the out-patient clinic. The differences between HI and PI periods are tested for each group separately.

	PERIOD 1	PERIOD 2
Group I	0.58±0.50 (PI)	0.68±0.48 (HI)
Group II	0.77±0.44 (HI)	0.38±0.51 (PI)

The insulin species that was used is given in parentheses.
The differences between periods are not statistically significant.

Body weight.

During the run-in period the percentual deviation of the ideal weight (DIW) increased by 1.6±2.9% ($p < 0.05$) for both groups together. No significant DIW changes were found during PI (group I: +0.1 ± 3.5% and group II: +1.1 ± 2.0%) nor during HI (group I: -1.1 ± 3.0 and group II: -0.6 ± 2.4%).

Insulin antibodies.

19 Of the 32 patients were IBA positive (12 in group I and 7 in group II).

During PI no significant changes in affinity nor capacity of the two binding sites could be demonstrated in both groups (table 7).

During HI no changes in affinity of both binding sites occurred in both groups but group II showed significant decreases of binding capacity of both binding sites (table 7). The decreases in binding capacities of group I during HI insulin were not statistically significant (table 7).

Since both HI periods were preceeded by treatment with PI (PI period in group I and run-in (=PI) period in group II), we analysed the change in binding parameters during HI for group I and II together. Only the binding capacity of the low affinity binding site decreased for both groups together (table 7). Figure 2 shows the change of binding capacity of the low affinity binding site during HI for each individual patient.

Discussion.

In several studies the efficacy of human and porcine insulin has been compared. A faster absorption of human insulin was found in healthy volunteers (8,9,10). But apparently this difference caused no change in glucose levels in most short and long term clinical studies in diabetic patients (3,4,6,16,23,24). However in these studies the insulin dose was variable, so that dose adjustments could annihilate small differences between human and porcine insulin.

We studied a group of patients, who injected insulin once a day. They were insulin dependent from the diagnosis on or had become insulin dependent after a period of treatment with oral hypoglycaemic drugs. Their glucose regulation as a group was not sufficient. To compare the efficacy of HI and PI we have chosen a study design in which the insulin dose was only adjusted in case of unexplained severe hypo- of hyperglycemias.

Table 7: Mean ± S.D. parameters of insulin antibodies in IBA positive patients at the start and the end of HI and PI periods in group I and II.

Group I		P PERIOD (N=12)		H PERIOD (N=11)	
	units	start	end	start	end
K1	nmol/l	0.021±0.036	0.028±0.059	0.030±0.061	0.015±0.010
Cap1	u/l	1.26±3.30	1.21±3.26	1.32±3.38	0.48 ±0.67
K2	nmol/l	6.57±8.53	5.76±7.14	6.14±7.35	2.91±1.84
cap2	U/l	21.19+25.83	11.48±11.80	12.26±12.03	9.69±8.83

Group II		H PERIOD (N=7)		P PERIOD (N=7)	
	units	start	end	start	end
K1	nmol/l	0.011±0.006	0.009±0.004	0.009±0.004	0.007±0.005
Cap1	U/l	0.21±0.07 *	0.13±0.06	0.13±0.06	0.10±0.05
K2	nmol/l	3.89±2.35	3.48±2.73	3.48±2.73	2.89±3.58
Cap2	U/l	5.37±3.27 *	3.10±2.33	3.10±2.33	4.81±6.51

H PERIOD of group I and II together (N=18)

	Units	Start		End
K1	nmol/l	0.023±0.049		0.013±0.008
Cap1	U/l	0.88±2.70		0.34±0.55
K2	nmol/l	5.26±6.03		3.13±2.25
Cap2	U/l	9.58±10.19	*	7.13±7.75

* $p < 0.05$

K1 and K2 are dissosiation constants and Cap 1 and Cap 2 binding capacities of first and second binding site of the insulin antibodies.

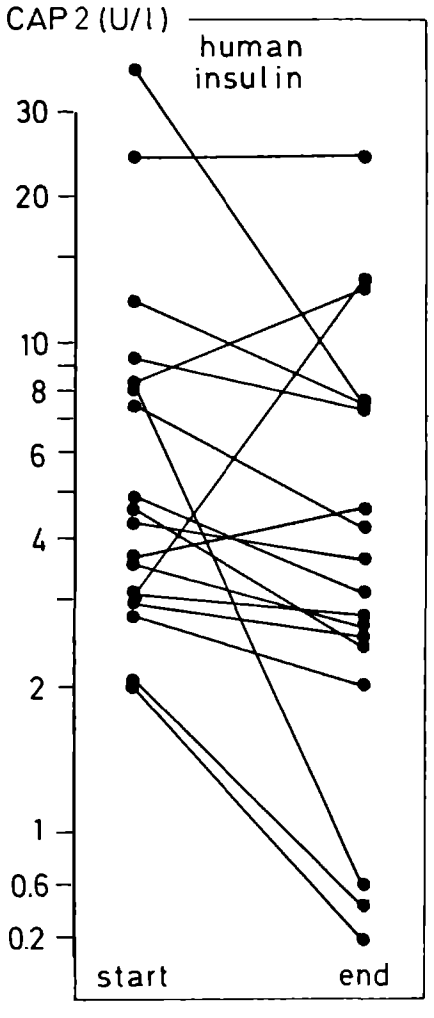

Figure 2: Changes of low affinity capapacity of insulin antibodies during human insulin. Group I and group II parameters are both included.

It is not easy to obtain a clear impression of the diabetes regulation in an out-patient clinic, because there is no single parameter that indicates mean blood glucose and variability of blood glucose. For the assessment of the mean metabolic control HbA1 levels were compared. The variability of the blood glucose was studied by means of glucosuria and home monitored blood glucoses. Glucosuria appears after the blood glucose has exceeded the renal threshold. This threshold however varies considerably between but also within patients (25). The blood glucose levels that are collected with strips at home have proved to be very reliable in recent studies (18), but represent only 5 points of time during 24 hours, thus giving an incomplete picture of the blood glucose regulation.

A small but significant increase of the mean HbA1 level was found only during human insulin in group II. In both groups glucosuria during HI exceeded the one during PI from 12 a.m. to 5 p.m. In group I a slightly higher home blood glucose was found at 12 a.m. during HI. Using a two way variance analysis of home blood glucoses the diurnal variation during PI exceeded the one during HI. Recapitulating these partially conflicting results, there might be a small difference in efficacy between HI and PI, but we believe it is clinically not important. The faster absorbtion of HI, found in short term studies (8,9,10), should have resulted in lower blood glucoses at 12 a.m. and higher blood glucoses at 8 a.m. during HI. We found however no difference in blood glucose at 8 a.m. and a higher blood glucose at 12 a.m. and a higher glucosuria from 12 a.m. to 6 p.m.

A second aspect of the study was to evaluate insulin antibody levels after changing from animal to HI. A major issue when comparing IBA levels is the assay method. It is not possible to compare the results between studies that use a different IBA assay. Using a method that describes the antibodies by the binding capacity of patient serum with a fixed insulin concentration, most of the studies found no difference (16,17), but some observed a decrease of insulin binding during HI (13). The second group of IBA assays describes insulin antibodies by two binding sites, each one having its own binding capacity. The only study using this kind of assay so far could not find a difference in antibodies after changing form PI to HI (13).

The IBA assay we used, describes the antibodies by two affinities and two capacities and takes into account a non specific binding for each plasma individually. We showed a decrease in IBA capacity of the low affinity binding site for the two groups together after 3 months of treatment with HI. In one group there was a significant decrease of the binding capacity of both binding sites. This decrease of IBA might have been more obvious if the period of treatment had been longer. Since we used an assay that assesses a non specific binding for each plasma individually and the study of Fineberg (13) used a constant non specific binding, this might be the reason why we did find differences and Fineberg did not. Obviously this decrease of IBA did not influence the clinical situation of the patients, using the same dose of insulin.

We conclude that HI is effective in the treatment of diabetic patients and that there are no clinically important differences between HI and PI. Insulin antibody levels in patients previously treated with animal insulins tend to decrease during treatment with HI within 3 months.

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CHAPTER 5

COMPARISON OF THE EFFICACY OF HUMAN AND PORCINE INSULIN IN THE TREATMENT OF DIABETIC KETOACIDOSIS.

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Abstract.

The efficacy of semisynthetic human (HI) and monocomponent porcine insulin (PI) in the treatment of diabetic ketoacidosis was compared in 10 (PI) versus 11 (HI) patients in a double blind randomized study. Insulin (i.v. 8u/hour), fluid replacement (NaCl 0.65% and glucose 5%) and KCl supplements were administered according to a fixed protocol. Glucose, potassium, sodium, creatinine, calcium, phosphate and free insulin concentrations were not significantly different at the start nor at any later time point. At the start mean ± S.D. of pH was 7.10 ± 0.14 in the HI group and 7.10 ± 0.12 in the PI group. The time to reach arbitrary values for pH, bicarbonate, base excess and B-OH-butyrate was shorter during human insulin, but the differences were not statistically significant. During human insulin the arbitrary value of 1.0 mmol/l of acetoacetate was reached faster than during PI (5.2 ± 2. 6 respectively 8.4 ± 0.9 hours, $p < 0.05$). The concentration of acetoacetate was significantly different between the two groups after 6 and 7 hours of insulin treatment (6 hours: HI 0.82 ± 0.50 mmol/l and PI 2.19 ± 1.65 mmol/l, $p < 0.05$; 7 hours: HI 0.51 ± 0.40 mmol/l and PI 1.74 ± 1.54 mmol/l, $p=0.05$). We conclude that the recovery from diabetic ketoacidosis during the treatment with HI might be slightly faster than during treatment with PI. If this difference is real it does not seem of much clinical importance.

Introduction.

The last few years highly purified human insulin (HI) is available for the treatment of insulin dependent diabetic patients. Small differences were found in studies comparing efficacy of intravenously administered human and porcine insulin (PI). In volunteers the insulin induced glucose consumption was significantly higher during HI than during PI (1, 2) and HI induced a smaller decrease in serum potassium (3, 4). In other studies no differences in potency of HI and PI were found in volunteers (5, 6, 7) or in insulin dependent diabetic patients (7, 8). Theoretically it is possible that these differences become more obvious in the treatment of the most severe acute form of diabetes, the diabetic ketoacidosis (DKA).

So far one study compared the efficacy of HI and PI in the treatment of diabetic ketoacidosis (9). The study was not randomized, nor double blind. The control group was treated in the months before and there was no fixed treatment protocol as far as insulin dose, fluid replacement and electrolyte supplements were concerned. Using this study design no difference in efficacy between the two insulins was found. Aim of our study was to compare the efficacy of HI and PI in the treatment of DKA. We performed a double blind, randomized, prospective clinical trial, with a fixed treatment protocol.

Subjects, materials and methods.

Design of the study.

Diagnosis of DKA was made on clinical grounds and confirmed by hyperglycaemia, ketonuria (Gluketur teststrips Boehringer) and an arterial pH below 7.25. After the diagnosis was confirmed patients were randomized for the double blind administration of HI or PI. Treatment was instituted according to a fixed protocol, of which the efficacy in severe ketoacidosis has been demonstrated earlier for PI (10).

1. NaCl 0.65% was used as infusion fluid. During the first 6.5 hours 5000 ml were infused, of which 1000 ml were administered in the first 30 minutes. After the arterial pH had reached 7.35, 1000 ml was administered every 4 hours. When the bloodsugar had reached a level of 12.0 mmol/l NaCl 0.65% was substituted by glucose 5%. Bicarbonate therapy was not used, unless the arterial pH was below 7.0 at admission and had not increased after 2 hours of treatment. This was the case in one patient who recieved HI.
2. 8 U of insulin per hour were infused i.v. continuously by pump after the result of the plasma potassium concentration had become available. The insulin used was monocomponent human or porcine Actrapid. The syringe containing insulin was filled with 1 ml(=40 U) Actrapid insulin, 10 ml pasteurised human plasma protein solution and 29 ml NaCl 0.9%.

3. If at any point of time the plasma potassium level was below 5.0 mmol/l, 15 mmol KCL were added to every 500 ml of infusion fluid.

During treatment venous blood samples were collected from a separate catheter every hour from time point zero, being the starting-point of insulin therapy, for the measurement of glucose, sodium, potassium, IRI (total + free), B-OH-butyrate (BOHB) and acetoacetate (ACAC). Arterial blood gas analysis was also performed every hour. At the start and every 4 hours after the start of treatment creatinine, calcium and phosphate were determined in venous blood samples.

This protocol was followed until the pH had reached a level of 7.35.

The study was approved by the ethical committee of our hospital and patients or relatives gave informed consent.

Patients.

Ten patients were treated with porcine insulin (PI) and eleven with human insulin (HI). Between these two groups there were no significant differences in sex, state of conciousness, age, duration of insulin therapy before DKA, basal levels in venous blood of glucose, BOBH, ACAC, creatinine, calcium, phosphate, sodium, potassium, and IRI. Also no differences were found in arterial pH, bicarbonate and base excess. In group PI, 1 out of 10 and in group HI, 3 out of 11 patients had not received insulin treatment before. This difference is not statistically significant. For the data see table 1. As appears from table 1 not all parameters were available at the start. After one hour of treatment however, all parameters were available for all patients and again no significant differences were found. The condition that precipitated DKA was a respiratory tract infection in 2 patients of group PI. In the other 8 patients of this group no cause was discovered. In group HI, respiratory tract infection was present in 1 patient, urinary tract infection in 2, enteric infection in 1, 2 patients had omitted insulin therapy and 1 patient had a severe acute pancreatitis. The cause of the ketoacidosis was not known in the other 4 patients. Two patients of group PI and 4 of group HI used other medications in addition to insulin, mostly antibiotics.

Table 1: Patients, biographical and biochemical data at the start of treatment. Data are given as mean ± S.D.

Insulin origin	Units	Porcine		Human	
N		10		11	
Consciousness abnormal		5		3	
Age	years	54.5±19.7		47.0±22.9	
Age range	years	15.3-79.5		14.3-77.7	
Duration of diabetes mellitus	years	16.7±12.7		19.7±13.2	
Treated with insulin before	number	9		8	
Duration of insulin therapy	years	13.6±14.0		19.6±11.2	
Other drugs than insulin	number	2		4	
Glucose	mmol/l	32.9±17.8	*	34.5±15.4	
Arterial pH		7.10±0.12		7.10±0.14	
BOHbutyrate (N)	mmol/l	8.94±2.67	(6)	7.89±2.35	(10)
Acetoacetate (N)	mmol/l	3.16±0.88	(6)	2.97±1.46	(10)
Bicarbonate	mmol/l	6.3±5.0		6.5±4.0	
Base excess	mmol/l	-21.8±6.6		-22.0±6.7	
Creatinine	µmol/l	254±134		224±85	
Calcium (N)	mmol/l	2.40±0.05	(4)	2.40±0.27	(8)
Fosfate (N)	mmol/l	1.50±0.24	(4)	1.60±0.36	(8)
Sodium	mmol/l	138±7		138±6	
Potassium	mmol/l	5.0±0.9		5.0±1.1	
Free insulin (N)	mU/l	11±9	(4)	10±6	(8)

* $p < 0.05$

Analytical methods.

Plasma glucose was analysed using the Hofman method (11). Arterial pH, bicarbonate and base excess were measured by a Corning 175 automatic blood gas analysis system. Venous blood was collected in ice-cooled tubes and extracted within 30 minutes by perchloric acid for the determination

of BOHB and ACAC by an enzymatic method (12). The Nakagawa (13) method was used for the determination of total and free IRI in venous serum. Routine laboratory methods were used for the other parameters.

Statistics.

Results are expressed as mean ± SD. For the analysis of 2 by 2 contingency tables the Fisher exact test was used. For other statistical analyses the Wilcoxon 2-sample test for unpaired observations was used. Regression was analysed by Pearsons method. After 7 hours of treatment so many patients had recovered from DKA that further statistical analysis of differences between PI and HI in the remaining patients was not possible anymore.

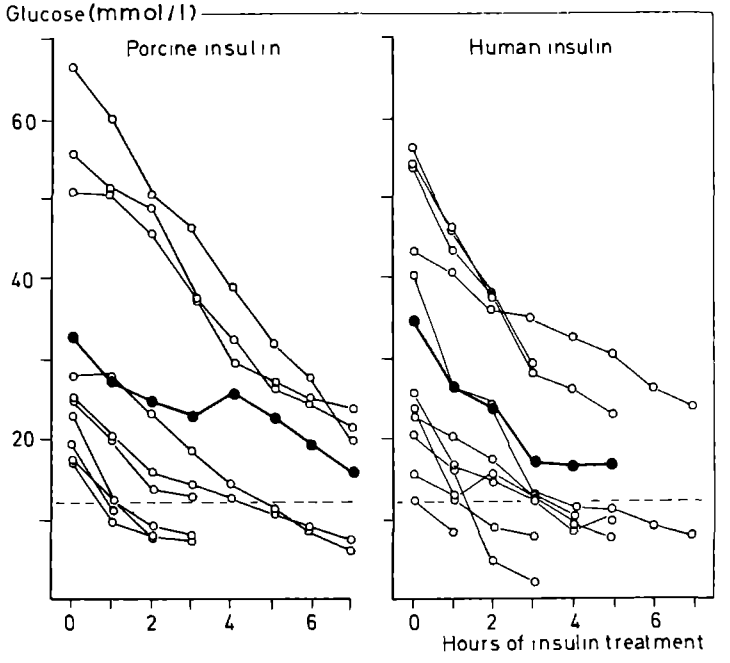

Figure 1: Mean (●) and individual (o) glucose levels during the first 7 hours from the start of PI and HI treatment untill the fluid infusion was changed from saline 0.65% to glucose 5% or the pH reached a level of 7.35. At no time point significant differences of mean glucose were found between PI and HI treated patients.

Results.

1. Complications.

During the treatment one patient became hypoglycaemic, because the 0.65% saline infusion was substituted too late by 5% glucose. This patient was treated with PI.

One patient, a 79 years old woman, died during the treatment. She was admitted to the hospital in an unconscious state with an arterial pH of 7.18 and a glucose level of 60.3 mmol/l. After the start of the protocol the pH recovered to 7.27 and the glucose had decreased to 35 mmol/l after 6 hours. She had become somnolent and complained about pain in the abdomen. After 7 hours the pH deteriorated again despite a decrease of BOBH and ACAC levels. 10 Hours after admission the patient died in shock. At autopsy a thrombosis of the arteria mesenterica superior and a massive infarction of the small gut was found. This patient was treated with HI. Her data have been included in the results. All other patients recovered from the DKA within 24 hours.

Table 2: Hours needed to reach the indicated arbitrary value of the parameters of diabetic ketoacidosis during the treatment with porcine and human insulin. Data are expressed as mean ± SD.

	Value	Porcine insulin	p	Human insulin
Glucose	12 mmol/l	4.7±3.5	N.S.	5.5±4.0
pH	7.30	8.3±2.0	N.S.	7.5±2.0
Bicarbonate	15 mmol/l	8.1±2.6	N.S.	6.1±1.6
Base excess	-10 mmol/l	10.5±8.8	N.S.	7.8±5.7
BOHbutyrate	1 mmol/l	9.2±1.0	N.S.	8.4±1.1
Acetoacetate	1 mmol/l	8.4±0.9	0.04	5.2±2.6

2. Plasma glucose concentration.

No difference in glucose level was found between the two groups at the start (table 1). During the first 7 hours the glucose concentrations decreased in both groups by the same rate, so that no significant differences could be found at any point of time (figure 1). As can be seen in figure 1 the individual glucose concentrations varied considerably. This interpatient variation did not differ significantly between the groups (variance analysis). The time necessary to reach a glucose level of 12.0 mmol/l was not different for the two groups (table 2).

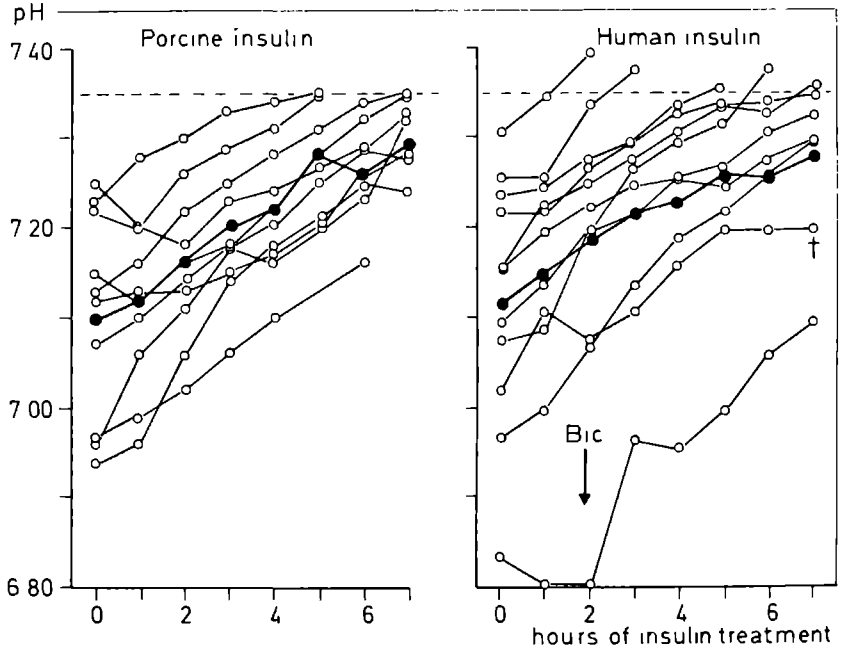

Figure 2: Mean (•) and individual (o) pH values during the first 7 hours from the start of PI and HI treatment untill the protocol was stopped. At no time point significant differences of mean pH were found between PI and HI treated patients. In the HI treated group 1 patient died in shock (†) and one patient received bicarbonate once (Bic).

3. Ketoacidosis.

At the start of the insulin therapy no significant differences in the parameters of ketoacidosis were found between the two groups (table 1). Figure 2 shows the effect of the therapy on the pH for the two groups. Only one patient (HI group) received once 100 ml $NaHCO_3$ 4.2%. No differences were found at any point of time between the two groups. The time needed to reach a pH of 7.30 was not significantly different for the two groups (table 2).

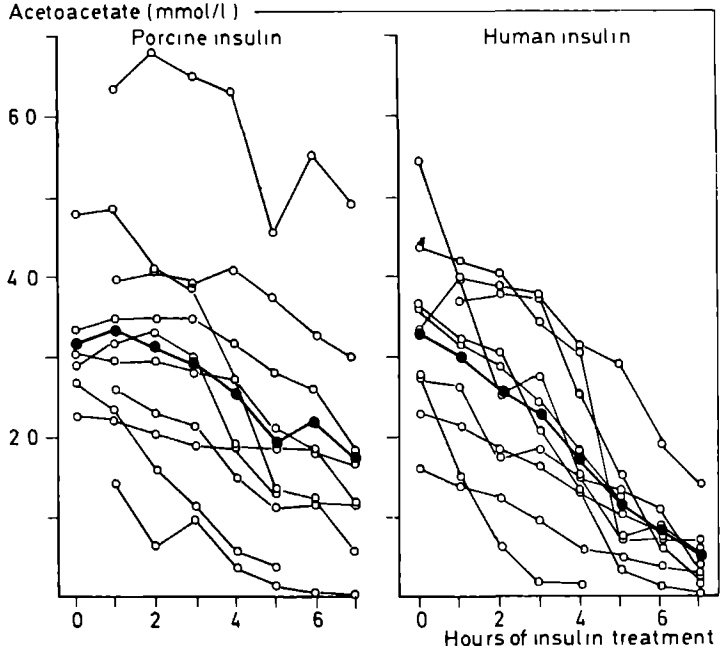

Figure 3: Mean (●) and individual (o) acetoacetate levels during the first 7 hours from the start of PI and HI treatment as long as this ketoacid was measured. After 6 ($p < 0.05$) and after 7 hours ($p=0.05$) mean acetoacetate levels of patients treated with HI were lower than the mean values of the patients treated with PI.

As was the case for pH, the time needed for bicarbonate, base excess, BOBH and ACAC to reach a certain arbitrary level, was in the HI group a little shorter than in the PI group, but this difference reached statistical significance only for ACAC (table 2). When we analysed the ACAC levels each hour the difference between group PI and HI became significant after 6 hours (2.19 ± 1.65 versus 0.82 ± 0.50, $p < 0.05$) and 7 hours (1.74 ± 1.54 versus 0.51 ± 0.40, $p=0.05$) (Fig. 3).

4. Potassium and sodium concentrations.

No significant differences of potassium and sodium levels were found at the onset of treatment (table 1). The sodium levels did not differ significantly between HI and PI treatment at any of the first 7 hours of treatment. After 3 hours of treatment the potassium level was 4.2 ± 0.6 mmol/l during HI (N=10) and 5.0 ± 0.7 mmol/l during PI (N=10) ($p=0.023$). No significant differences in potassium concentration were found at other times.

5. Calcium, phosphate and creatinine concentrations.

No significant differences between group HI and PI were found for these parameters at the start (table 1). Neither were significant differences found after 4, 8 and 24-48 hours of treatment (figure 4). After 24-48 hours the creatinine concentration was normalized almost completely. Phosphate concentration were still very low after 24-48 hours.

6. IRI.

Free IRI was measured at the start in 4 out of 10 patients in group PI and 8 out of 11 patients in group HI. These free IRI levels were not significantly different (table 1). In all patients free IRI levels were measured from 1 hour after the start of insulin therapy on. As shown in figure 5 no differences in free insulin were found at any of the first 7 hours between HI and PI. There was a considerable individual variation of free IRI concentrations, although all patients received 8 U of insulin per hour. For every patient a mean bound and free insulin concentration

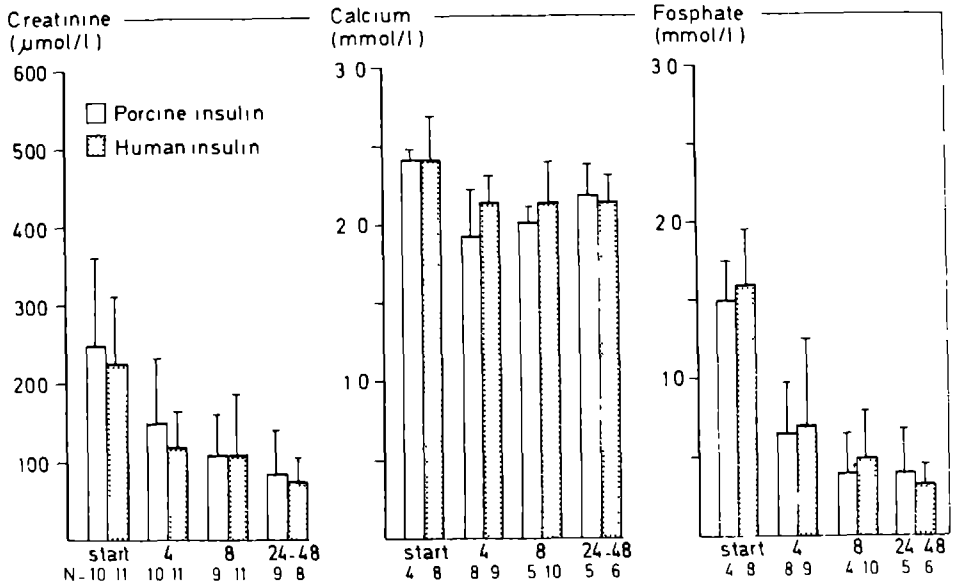

Figure 4: Mean ± SD creatinine, calcium and phosphate levels at the start and after 4, 8 and 24-48 hours of treatment with PI or HI. At no time point significant differences between PI and HI treated patients were found.

during the first 7 hours was calculated. Nor for the HI group (R=-0.26, NS) nor for the PI group (R=-0.02, NS) nor for both groups together (R=-0.13, NS) there was a significant correlation between mean free and mean bound insulin.

No significant correlation was found between mean free IRI concentration and increase of pH per hour for group HI (R=0.02, NS), for group PI (R=-0.29, NS) and for HI and PI groups together (R=-0.03, NS). The patients with the lowest mean free IRI had levels of 39 (PI) and 42 (HI) mU/l. Their recovery was not delayed.

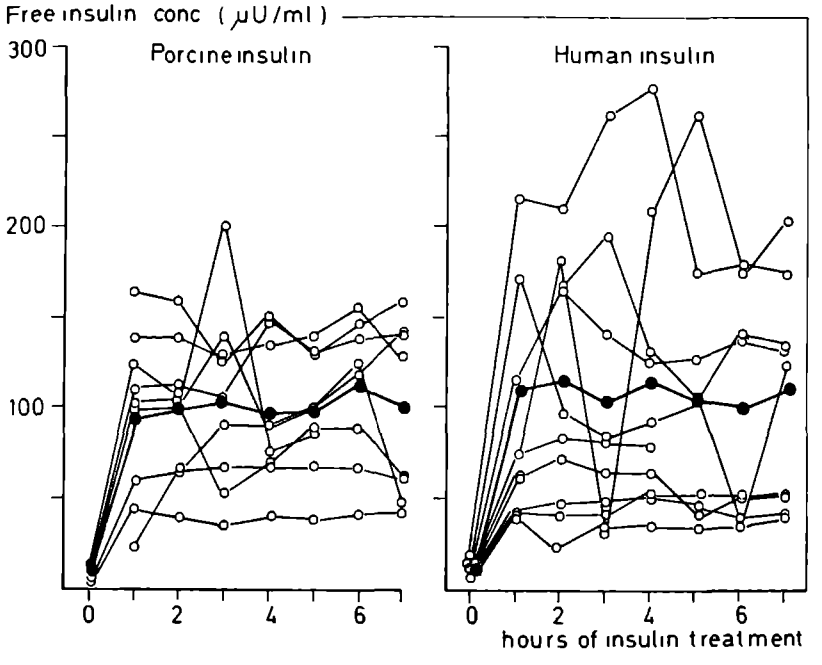

Figure 5: Mean (●) and individual (o) free insulin levels during the first 7 hours of treatment with PI and HI. Figures are given untill the protocol was ended. At no time point significant differences between PI and HI treated patients were found.

Discussion.

This study compared the efficacy of monocomponent HI and PI in the treatment of diabetic ketoacidosis. The rate of normalization of glucose, pH, bicarbonate, base excess and BOBH was the same during HI and PI. No differences were found in the levels of calcium, phosphate and creatinine after 0, 4, 8 and 24-48 hours. Serum phosphate was still very low after 24-48 hours as reported before (14). The mean concentration of ACAC decreased faster during HI. When we looked closer at the data all parameters of ketoacidosis tended to improve faster during HI, but the only significant difference was the rate of decrease of ACAC. Therefore there could be an overall faster improvement of DKA during HI. A slight differ-

ence in the rate of recovery between the two groups could be connected with a dissimilarity before the start of treatment. We feel that we may conclude from the data of table 1 that the condition of the patients in the PI group was not worse than in the HI group. There were no significant differences in the parameters of metabolic derangement. Admittedly, BOBH and ACAC were a little higher in the PI group, but pH and base excess were the same and glucose was slightly higher in the HI group. Also there was no difference in age between the groups.

Only one study compared the efficacy of human and porcine insulin during the treatment of DKA so far (9). No differences were found between the effects of HI and PI insulin. However this study was not randomized, nor double blind. The insulin infusion rate was not kept constant and no fixed protocol was used for fluid and electrolyte supplements. Moreover, BOBH and ACAC were not measured.

Clark et al. (15) infused intralipid and compared the antiketogenic effects of HI and PI in 9 healthy subjects. They found significantly lower levels of BOBH during infusion of HI and no difference in ACAC level. These findings point in the same direction as our results.

In several studies high dose and low dose infusion of insulin proved to be equally effective in the treatment of DKA (10, 16). Hypoglycaemias however occurred more frequently during high dose insulin infusion. In our study 8 U of insulin per hour were infused. At admission insulin levels were low. During treatment there was a considerable variation of free insulin concentrations between patients that could not be explained by insulin binding by antibodies, because we did not find a negative correlation between free and bound insulin. The lowest mean steady state free insulin concentration was 39 mU/l, indicating that an insulin infusion still lower than 8 U per hour, may result in insulin levels that are suboptimal for the treatment of DKA, for it has been shown that glucose uptake in muscle takes place between insulin levels of 20 and 200 mU/l (17). In our study no systematic difference was found between the steady state levels of free insulin in the PI and HI groups which is in agreement with published data about the pharmacokinetics of both kinds of insulin (2, 5, 6, 7, 18).

We conclude that human insulin is effective in the treatment of DKA. The recovery from DKA during treatment with HI might be slightly faster than

during treatment with PI. If this difference is real, it seems of not much clinical relevance.

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Summary.

The summary intends to give information to those who are interested in only one or a few chapters and to those who have not the time to read the thesis in detail.

In chapter 1 the production process of semisynthetic and biosynthetic human insulin is described. Subsequently clinical research that has been performed so far on human insulin is reviewed. Comparing porcine and human insulin most studies found no difference in efficacy, but in some a faster action of insulin after subcutaneous injection has been described. Although the hypoglycaemic effect of human insulin was the same as that of porcine insulin, one study found a less pronounced reaction of counterregulatory hormones after human insulin. This was not confirmed by several other studies. Whether or not human insulin is more effective than porcine insulin in the treatment of diabetic ketoacidosis is not clear, so far. When human insulin is used for at least 6 months it seems less immunogenic than porcine insulin in newly diagnosed diabetic patients. If human insulin is also less immunogenic in patients previously treated with highly purified porcine insulin has not been clearly shown yet.
The different assays for insulin antibodies are described in the following paragraph. Since these methods are fundamentally different, it is not possible to compare clinical studies on insulin antibodies. Besides these assay problems, the clinical relevance of insulin antibodies has not been completely established. The presence of IBA may be related to rare cases of allergy and may be accompanied by a higher incidence of hypoglycaemias and a worse control of blood sugar regulation than in the absence of antibodies. It has also been suggested that insulin antibodies are related to microvascular disease. Patients that do not develop insulin antibodies have longer remissions after the start of insulin treatment.

The insulin antibody assay that was used in our studies is described in chapter 2. The binding process of insulin by insulin antibodies is described by two different groups of binding sites, each having its own affinity and capacity. The non specific binding of insulin in the assay

was estimated as a distinct parameter for each individual plasma. From 19 insulin antibody positive patients plasma was collected every 3 months, during a period of 9 months. The assay was performed in 4 runs. Plasmas of each individual patient were always analysed in the same run of the assay. In all four runs intra-patient variance of non specific binding proved to be smaller than between-patient variance. This indicates that this non specific binding is patient dependent and therefore should be estimated for each patient individually.

The efficacy, the immunogenicity and the effect on residual B-cell function of human and porcine insulin in newly diagnosed type I diabetic patients is described in chapter 3. In this randomized, double blind, prospective study no clear differences between human and porcine insulin were found. The only difference with a possible clinical importance was that during treatment with human insulin the between days variance of home blood glucose was smaller than during porcine insulin.
In 5 patients treated with human insulin and in 7 patients treated with porcine insulin, insulin binding antibodies were analysed from 0 to 18 months, with an interval of 6 months. No differences between the two groups were found. However, our conclusions on this point must be guarded, because of the rather small number of patients, that completed this study so far.

In chapter 4 a double blind, randomized, prospective, cross-over study is described in which the efficacy and immunogenicity of human and porcine insulin have been compared in patients that were previously treated with animal insulins. 32 Patients completed the study. All the patients used long-acting insulin once a day, Monotard insulin with or without Actrapid insulin. After a run-in period of 3 months, in which the patients used porcine insulin only, they were treated with human or porcine insulin for three months. After this time the insulin species was switched for another three months. In the group that received human insulin first a slight but significant increase of HbA1 was observed during human insulin. In the group that received porcine insulin first the mean blood glucose during human insulin at 12 a.m. exceeded the one during porcine insulin. In both groups the glucosuria from 12 a.m. to 6 p.m. was higher

during human insulin. Although the blood glucose concentration was only different at 12 a.m. in one group, the diurnal variance of these glucose concentrations during porcine insulin exceeded the diurnal variance during human insulin in both groups together. The binding capacity of the low affinity antibodies decreased during human insulin and did not change significantly during porcine insulin.
So, small differences in metabolic control could be demonstrated between human and porcine insulin. Insulin antibody concentration decreased during 3 months of treatment with human insulin, indicating a lower immunogenicity compared to porcine insulin.

A study comparing the efficacy of semisynthetic human and monocomponent porcine insulin in the treatment of diabetic ketoacidosis is described in chapter 5. Patients presenting with an arterial pH equal or below 7.25 were randomly assigned to a double blind treatment with human (N=11) or porcine (N=9) insulin. The fixed treatment protocol consisted of 8 U/hour of insulin intravenously by a continuously running pump, fluid replacement by NaCl 0.65% and glucose 5% and potassium supplements when the plasma potassium level was found to be below 5.0 mmol/l. A number of parameters was measured every hour. No significant differences were found at any time point for glucose, pH, bicarbonate, base excess and B-OH-butyrate. The time of recovery to arbitrary levels of pH, bicarbonate, base excess and B-OH-butyrate was not significantly shorter during human insulin. The only significant difference found, was the shorter recovery time for acetoacetate. After 6 and 7 hours of treatment the plasma concentrations of acetoacetate were significantly lower during human insulin. From these data we conclude that in the experimental circumstances described above, human insulin is slightly more effective in the treatment of ketosis in diabetic ketoacidosis.

Summarizing all the results of the clinical studies, in which a lot of parameters for diabetic control were analysed, human insulin differs only slightly from porcine insulin as far as efficacy is concerned. These small differences were only seen in diabetic patients who used the same dose of human and porcine insulin. When the aim was to achieve normoglycaemia with a free insulin dose regimen, human and porcine insulin

proved to be equally effective. Both in newly diagnosed patients and in patients who were previously treated with animal insulin, the variance of home blood glucose concentrations during porcine insulin exceeded the variance during human insulin. In patients who were treated with monocomponent porcine insulin before, human insulin treatment resulted in a decrease of insulin binding antibodies within 3 months. In a smaller number of newly diagnosed patients no differences in insulin antibody parameters were found between human and porcine insulin treatment after a period up to 18 months. Human insulin proved to be slightly more effective in the treatment of ketosis in patients who presented with ketoacidosis. Except for the variance of home blood glucose concentrations, that was not assessed in other studies, our results on efficacy of human insulin are in concordance with recently published studies. However, the decrease of insulin binding antibodies within 3 months in patients previously treated with porcine insulin was not found by other investigators. This difference could perhaps be explained by the fact that we used a more thorough assay for insulin binding antibodies.

Overlooking studies from other authors and our studies, we may conclude that the probable replacement of animal insulins by human insulin within the next 10 years, will cause no problems for physicians and patients, but the question whether this replacement will be a benefit from the clinical point of view still remains open.

Samenvatting.

Gepoogd is in de Nederlandse samenvatting medische en wetenschappelijke "geheimtaal" zoveel mogelijk te vermijden zodat deze ook voor niet ingewijden enigszins leesbaar is.

In hoofdstuk 1 wordt in het kort beschreven wat er tot nu toe bekend is over het gebruik van menselijke insuline bij de behandeling van suikerziekte. In het verleden werd steeds van dieren afkomstige insuline (rund, varken) gebruikt. Dierlijke insuline is in geringe mate anders van chemische samenstelling dan menselijke insuline. Sinds kort zijn er 2 manieren in gebruik om menselijke insuline te maken: door chemische verandering van varkensinsuline en door manipulatie van materiaal waarin de erfelijke eigenschappen liggen verankerd (DNA). Deze twee productiemethoden worden in hoofdstuk 1 besproken. Bij de mens wordt insuline onder de huid gespoten om van daar uit in het bloed terecht te komen waar de voornaamste functie het verlagen van het suikergehalte is. Sommige onderzoekers vonden dat de menseljke insuline sneller in het bloed komt dan varkensinsuline en dat het bloedsuikergehalte ook sneller daalt. Een groter aantal andere onderzoekers vond geen verschil. Wanneer er te veel insuline en daardoor te weinig suiker in het bloed is, krijgt een diabetespatient een zeer onaangename sensatie, een zogenaamde hypoglycaemie ("hypo"). Deze sensatie wordt mede veroorzaakt door bepaalde stoffen (hormonen) die als reactie op het lage bloedsuikergehalte vrijkomen. In één studie was de uitstorting van deze bloedsuikerverhogende stoffen na een te laag bloedsuikergehalte minder groot na menselijke dan na varkensinsuline. Verscheidene studies, die dit verschil ook zochten, hebben het niet kunnen aantonen. Wanneer een diabetespatient, die afhankelijk is van insuline, te lang te weinig insuline krijgt toegediend treedt er een situatie op waarbij het bloedsuikergehalte zeer hoog kan worden en het bloed zuur wordt. Deze toestand, diabetische ketoacidose genaamd, leidt, indien er niet wordt ingegrepen, tot de dood. Indien de werkaamheid van menselijke en varkensinsuline verschillend is, zou dit bij de behandeling van deze ernstigste vorm van diabetes duidelijk kunnen worden. In één studie werd dit punt onderzocht, maar er werd geen verschil gevonden. Op de organisatie van die studie is echter nogal wat kritiek uit te oefenen

zodat de vraag, welke van de twee insulinesoorten effectiever is bij de behandeling van ernstige diabetische ontregelingen, nog niet beantwoord is.
Wanneer men bij de mens lichaamsvreemde stoffen inspuit zal hij hierop reageren met het maken van antistoffen, die zich aan deze vreemde stoffen binden, waarna ze opgeruimd kunnen worden. Het is bekend dat deze antistoffen ook tegen dierlijke insuline gemaakt worden en dat een merendeel van de diabetespatienten ze bezit. Aangezien varkensinsuline meer lijkt op menselijk insuline dan runderinsuline worden hiertegen ook minder antistoffen gemaakt. Daar menselijke insuline, dat gemaakt wordt op de twee bovenstaande manieren, gelijk is aan de stof die in de alvleesklier van de mens wordt gemaakt, zou het lichaam deze insuline niet als vreemd moeten beschouwen. In verschillende onderzoeken is echter al aangetoond dat ook tegen menselijke insuline antilichamen worden gemaakt. Wel bleek in sommige studies dat er minder antilichamen tegen menselijke dan tegen varkensinsuline worden gemaakt. Andere studies konden dit niet bevestigen. Bij patienten met grote hoeveelheden antistoffen tegen insuline kan een insulineongevoeligheid ontstaan, dat wil zeggen dat ze zeer grote hoeveelheden insuline moeten gebruiken om het bloedsuikergehalte omlaag te krijgen. De betekenis van geringere hoeveelheden antistoffen voor de patient is niet duidelijk. Mogelijk hebben patienten met insuline antistoffen meer kans op hypoglycaemien, is hun bloedsuikergehalte minder goed in te stellen en hebben ze meer kans op bloedvatafwijkingen op de lange duur.

In hoofdstuk 2 is de methode van insuline antilichaambepaling beschreven. De manier waarop insuline door de antilichamen wordt gebonden is nogal ingewikkeld. Het bindingsproces kan beschreven worden door een getal, dat de gretigheid van de antilichamen tot binding (affiniteit) aangeeft, en een getal, dat aangeeft hoeveel er in totaal kan worden gebonden (capaciteit). Insuline-antistoffen blijken uit twee groepen te bestaan: de eerste heeft een grote affiniteit, maar een lage capaciteit en de tweede heeft een kleine affiniteit, maar een grote capaciteit. Naast deze binding aan antistoffen wordt insuline ook nog aan andere stoffen gebonden (niet-specifieke binding). Over het algemeen wordt aangenomen dat deze niet-specifieke binding kunstmatig is en wordt veroor-

zaakt door de methode die in het laboratorium gebruikt wordt om antistoffen te bepalen. Om nu specifieke binding te berekenen, wordt van de totale binding de niet-specifieke binding, die voor alle bepalingen hetzelfde is, afgetrokken. Bij onze manier van antistofbepaling werd voor ieder apart bloedmonster naast de affiniteit en capaciteit van de twee soorten antilichamen ook de niet-specifieke binding bepaald. Na bewerking van de uitkomsten bleek dat de hoeveelheid niet-specifieke binding niet alleen afhangt van de laboratoriummethode, maar ook van de patient. Dit betekent dat deze laatste voor iedere patient bepaald moet worden en dat niet een vaste hoeveelheid binding van iedere totale binding mag worden afgetrokken om de specifieke te berekenen.

In hoofdstuk 3 wordt een studie beschreven waarin de effectiviteit en het antilichaam-opwekkend effect (antigeniciteit) van varkens- en menselijke insuline vergeleken werden, bij patienten die insuline afhankelijk waren en tevoren nog niet met insuline behandeld waren. Hiervoor werden 11 patienten met varkens- en 9 patienten met menselijk insuline behandeld. De regulering van de bloedsuikers was in beide groepen gelijk. Het enige verschil dat werd gevonden was dat het bloedsuikergehalte van de ene op de andere dag minder varieerde tijdens menselijk insuline. Bij 5 patienten die behandeld waren met menselijk insuline en bij 7 die behandeld waren met varkensinsuline werden op gezette tijden insuline antilichamen bepaald. Na 6, 12, 15 en 18 maanden waren er geen verschillen wat betreft insuline-antilichamen tussen deze 2 groepen.

In hoofdstuk 4 wordt een studie beschreven waarin de effectiviteit en de antigeniciteit van varkens- en menselijke insuline wordt vergeleken bij 32 patienten die tevoren dierlijke insuline inspoten. Hiertoe kregen de patienten eerst 3 maanden varkensinsuline en vervolgens "dubbelblind", dat wil zeggen zonder dat de patient of de behandelend arts wisten welke insulinesoort het was, 3 maanden varkens- en daarna 3 maanden menselijk insuline (groep 1) of in omgekeerde volgorde (groep 2). De hoeveelheid insuline die de mensen eenmaal per dag spoten werd zo weinig mogelijk veranderd. In groep 1 bleek het gemiddelde bloedsuikergehalte om 12 uur 's middags tijdens menselijk insuline hoger te zijn. In groep 2 bleek het gemiddelde bloedsuikergehalte, over een periode van ongeveer 6 weken

gemeten, in geringe mate toe te nemen tijdens menselijk insuline. In beide groepen was de hoeveelheid suiker in de urine van 12 tot 18 uur hoger tijdens menselijk insuline. 19 Patienten hadden insuline antilichamen. Gedurende behandeling met menselijk insuline daalde de capaciteit van de groep antilichamen met lage affiniteit.

In hoofdstuk 5 wordt een studie beschreven waarin de effectiviteit van varkens- en menselijk insuline wordt vergeleken bij de behandeling van diabetische ketoacidose. 11 Patienten werden met menselijk en 10 met varkensinuline behandeld. Behalve wat betreft het verschil in insulinesoort was de behandeling gelijk voor beide groepen. De verbetering van de zuurgraad en van het bloedsuikergehalte was niet verschillend bij beide groepen. Tijdens een diabetische ketoacidose wordt de verzuring van het bloed grotendeels veroorzaakt door 2 zogenaamde ketozuren: acetylazijnzuur en B-hydroxyboterzuur. Het verdwijnen van acetylazijnzuur was duidelijk sneller tijdens menselijk insuline. Bij onze manier van behandelen bleek menselijk insuline dus iets effectiever te zijn dan varkensinsuline bij de behandeling van diabetische ketoacidose.

Wanneer we de resultaten van de drie studies, waarin wij menselijke en varkensinsuline vergeleken bij de behandeling van diabetespatienten samenvatten, blijkt dat er geen grote verschillen zijn. De werkzaamheid van menselijk insuline is iets anders, zonder dat dit betekenis voor de patienten heeft. Bij mensen, die tevoren met dierlijke insuline werden behandeld, blijkt menselijke insuline binnen 3 maanden een vermindering van de antistoffen te veroorzaken. Mogelijk is menselijke insuline effectiever dan varkensinsuline bij de behandeling van diabetische ketoacidose. Gezien studies in de literatuur en de resultaten van ons onderzoek zal de vervanging van dierlijke door menselijke insuline gedurende de komende 10 jaar geen problemen opleveren. Het blijft nog de vraag of deze vervanging iets gunstigs oplevert voor de patiënten.

Woorden van dank.

Allereerst wil ik alle patienten die aan de verschillende studies hebben deelgenomen danken voor hun enthousiaste medewerking. Dankzij hun inzet konden de vele gegevens verzameld worden.
De "suikerzusters" van de polikliniek bepaalden ontelbare keren het glucosegehalte in urinemonsters en even vaak werden bij patienten bloed monsters afgenomen. Ook de verpleegkundigen van de klinische afdelingen B40 en B60 wil ik danken voor de steeds enthousiaste hulp die zij, evenals de verpleegkundigen op de polikliniek, boden bij het instrueren van nieuwe diabetespatienten en niet te vergeten voor de assistentie die zij, zowel overdag, maar vooral ook 's nachts, boden bij de vele behandelingen voor diabetische ketoacidose.
Een grote hoeveelheid laboratoriumwerk is verzet door R. Hermsen. Hem en ook de andere medewerkers van het laboratorium voor Chemische en Experimentele Endocrinologie (hoofd Prof.dr. T. Benraad) wil ik danken voor de vele bepalingen die verricht zijn en voor de hulp bij het beschrijven van de insuline antilichaamassay. De routinebepalingen werden nauwkeurig als altijd verricht op het laboratorium van Inwendige Geneeskunde (hoofd Prof.dr. A.P. Jansen).
De statistische analyse vond plaats met accurate medewerking van de Mathematisch Statistische Adviesafdeling (hoofd Dr. Ph. van Elteren). Dankzij deze hulp is mijn inzicht in de statistische methodieken, hopelijk blijvend, verdiept.
De heer G. van Lingen van de afdeling Pharmacologie (hoofd Prof.dr. J.M. van Rossum) maakte het computermodel ter beschrijving van pharmaconreceptor interactie geschikt voor de beschrijving van insulinebindende antilichamen. Naast het mogelijk maken van de bestudering van niet-specifieke binding bij de bepaling van insulinebindende antilichamen, heeft het gebruik van dit programma mijn interesse voor het gebruik van computers gewekt.
Het aselect indelen van de patienten en het dubbelblind verstrekken van de insulinepreparaten is zeer accuraat verricht door de medewerksters van de Apotheek (hoofd Prof.dr. E. van der Kleyn).
Meerdere malen is een beroep gedaan op Drs. E. de Graaff en later Drs. S. Bakker van de Medische Bibliotheek, die steeds weer nieuwe literatuur uit de computer toverden.

De collega-artsen van one afdeling waren steeds alert op de diagnose nieuwe type I diabetes en diabetische ketoacidose en fungeerden als voor-uitgeschoven posten om de studies te "vullen". Hiervoor en voor het interesse dat zij steeds getoond hebben in mijn werk wil ik hen danken.
De heer C.P. Nicolasen van de afdeling Medische Illustratie heeft met vakmanschap de figuren en schema's getekend. Deze tekeningen werden door de afdeling Medische Fotografie (hoofd. A. Reynen) op de gevoelige plaat vastgelegd.
De steun, zowel immaterieel als financieel, die ik van NOVO Nederland heb gekregen, was steeds adequaat en maakte een soepel verloop van de studie mogelijk. Op deze plaats wil ik dan ook drs. C. Houtzagers en de heren J. van Veen en B. Spaay danken voor de altijd prettige samenwerking. Dr. L.G. Heding en Dr. I. Jensen van NOVO Denemarken wil ik danken voor de kritische inbreng bij het bespreken van de resultaten van de verschillende studies.
Last but not least wil ik mijn vrouw Ien Creusen bedanken voor het met veel geduld verrichten van de tekstverwerking en het bijstaan in de vaak drukke tijden. Het drukklaar maken van dit proefschrift werd verzorgd door Mevr. D. Graven, bibliothecaresse van de Kliniek voor Inwendige Ziekten.

Curriculum vitae.

De schrijver van dit proefschrift werd op 23-09-51 te Sittard geboren. Hij legde het eindexamen gymnasium-B in 1969 op het Bischoppelijk College te Sittard met goed gevolg af, waarna hij in hetzelfde jaar aan zijn medische studie begon aan de Katholieke Universiteit te Nijmegen. Het doctoraalexamen werd in 1975 en het artsexamen in 1977 behaald. Na te hebben voldaan aan zijn militaire dienstplicht, werd in 1978 begonnen aan de specialistenopleiding op de afdeling Interne Geneeskunde van het St. Radboudiekenhuis te Nijmegen (hoofd destijds Prof.dr. C.L.H. Majoor, huidig hoofd Prof.dr. A. van 't Laar). In juni 1981 begon hij op de afdeling Algemeen Interne Geneeskunde van het St. Radboudziekenhuis met de studies die tot dit proefschrift geleid hebben. In 1983 werd hij ingeschreven als internist. Momenteel is hij werkzaam op de afdeling Algemeen Interne Geneeskunde. Hij is getrouwd met Ien Creusen en heeft twee dochters, Anke en Irith.

STELLINGEN

1. Voor de behandeling van insuline-afhankelijke diabetes is varkensinsuline even effectief als menselijke insuline.

2. De bindingscapaciteit van insulinebindende antilichamen met lage affiniteit, die ontstaan tijdens behandeling met dierlijke insuline, neemt af wanneer de patiënten vervolgens met menselijke insuline behandeld worden, maar dit geringe verschil in immunogene eigenschappen heeft waarschijnlijk geen klinische betekenis.

3. Bij de behandeling van diabetische keto-acidose is een intraveneuze toediening van 8 eenheden insuline per uur voldoende, ook als er insulinebindende antilichamen zijn.

4. De niet specifieke binding van insuline in een "equilibrium binding assay" voor insulinebindende antilichamen is niet alleen afhankelijk van de bepalingscondities maar ook van individuele plasmafactoren.

5. Ook bij diabetespatiënten die intensief begeleid worden, resulteert het eenmaal daags injiceren van langwerkende insuline meestal niet in een acceptabele metabole controle.

6. De toename van de incidentie van cardiovasculaire accidenten door roken bij patiënten met een lichte hypertensie is groter dan de afname van de incidentie door medicamenteuze therapie.

   MRC working party: MRC trial of treatment of mild hypertension: principal results. Brit. Med. J. 1985; 291: 97-104.

7. Bij slecht ingestelde diabetespatiënten draagt niet-enzymatische glucosylering van LDL-apoB, met als gevolg een vertraagde klaring en verhoogde spiegels van LDL in het bloed, mogelijk bij tot de vroegtijdige ontwikkeling van atherosclerose.

   Steinbrecher, W.P. and Witztum, J.L.: Glucosylation of low density lipoproteins to an extent comparable to that seen in diabets slows their catabolism. Diabetes 1984; 33: 130-4.

8. Grote VDL-partikels (30-150 nm in diameter) worden evenals chylomicrons direct uit de circulatie geklaard zonder omzetting tot LDL.

   Stalenhoef, A.F.H., Malloy, M.J., Kane, J.P. and Havel, R.J.: Metabolism of apolipoproteins B-48 and B-100 of triglyceride-rich lipoproteins in normal and lipoprotein lipase-deficient humans. Proc. Natl. Acad. Sci. USA 1984; 81: 1839-43.

9. Fysiotherapie bij primaire pneumonie heeft geen gunstig effect op de genezing.

   Britton, S., Bejstedt, M., and Vedin, L.: Chest physiotherapy in primary pneumonia. Brit.Med.J. 1985; 290: 1703-4.

10. Het invoeren van een algemeen co-assistentschap (ALCO) zal de waarde van de daarop volgende klinische co-assistenschappen doen toenemen.

11. Tijdens de klinische co-assistentschappen dienen tussentijdse evaluaties van ervaring en kundigheid van de student te worden ingevoerd.

    PKO projectgroep: Een evaluatie van het coschap interne geneeskunde 1984.

12. De gewoonte om te applaudiseren voor de piloot na een geslaagde vlucht is een uiting van een gebrek aan vertrouwen in de veiligheid van het vliegverkeer.

Nijmegen, 12 september 1985 Fred Storms